IDENTIFICATION OF A COLD AGGLUTININ AS A β GLOBULIN

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The introduction of the anti-human globulin test (Coombs, Mourant and Race, 1945) has made the detection of a globulin adsorbed to the red cells of patients with haemolytic syndromes very simple.

One of the results of the investigation of these syndromes has been the discovery that a positive Coombs test may be due to different varieties of adsorbed globulin. Coombs and Mourant (1947) found that the component of their anti-globulin serum which reacted with red cells coated with Rh antibody was probably a gamma globulin and this has since been confirmed by others (Dacie, 1951, and Vaughan and Waller, 1954). Dacie (1951) showed that the warm agglutinins present in patients with acquired haemolytic anaemia were also probably gamma globulins. However, serological differences between red cells sensitized with Rh antibodies, blood group A antibodies, non-specific cold agglutinins and warm agglutinins present in acquired haemolytic anaemia have been reported (Crawford and Mollison, 1951). Furthermore, Vaughan and Waller (1954) have shown that the substances coating the red cells from two patients with acquired haemolytic anaemia were not gamma globulins. Evidence that the non-specific cold agglutinin is a different type of globulin to that causing anti-D sensitization has been presented by Dacie (1951) and Renton (1952) and recently auto- and iso-immune antibodies have been differentiated *in vitro* by the use of heparin (Roth, 1954) and protamine and trypsin (Roth and Framin, 1954).

The present paper describes the identification of a cold agglutinin present in a patient with acquired haemolytic anaemia of unknown aetiology by the use of the gel-diffusion technique of Ouchterlony (1953).

METHODS.

Albumin, α_2, β, and γ globulins were obtained by eluting with normal saline the appropriately identified zones of paper electrophoretograms of normal serum and of the patient's serum (Wunderly *et al.*, 1953). The patient's serum was obtained by allowing the whole blood to clot in a water bath and then centrifuging for 6 minutes at 5000 r.p.m., both being

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carried out at 37° C. to retain the cold agglutinins in the serum. Eluents were stored, when necessary, in a cold room at 10° C.

The *cold agglutinin* was detached from the patient's red cells by the following modification of the method of Aubert and Brendemoen (1949). 9 ml. of the patient's blood were collected into a centrifuge tube containing 1 ml. of Wintrobe's oxalate solution and thoroughly mixed. After the mixture had been left for 12 hours in a cold room at 10° C., it was centrifuged at the same temperature and the plasma was discarded. The red cells were washed three times with 10 ml. of cold normal saline. The washed red cells with the attached agglutinin were suspended in 2 ml. of normal saline and incubated for 15 minutes at 37° C. Finally the saline containing the cold agglutinin was separated from the red cells by centrifuging at 37° C.

Coombs anti-human globulin rabbit serum was used as supplied by the Commonwealth Serum Laboratories.

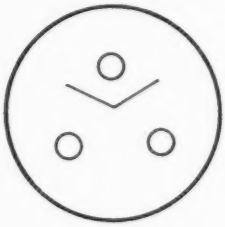

Fig. 1.

1. Cup at apex contains antibody to a known antigen.
2. Cup at right base contains known antigen.
3. Cup at left base contains unknown antigen.

A single, smooth, continuous band of precipitate has formed, between the cups containing the antigens and that containing the antibody and this constitutes a reaction of identity between the antigens.

Ouchterlony's method (Oudin, 1952) of *gel-diffusion* was employed to identify the cold agglutinin. Purified agar (supplied by Davis Gelatine Co.) was made up to a concentration of 1·5 p.c. w/v in normal saline, methiolate 1/10,000 was added as a preservative and the hot agar solution was poured into petrie dishes 5 cm. in diameter. Shallow cups were made in the agar at the angles of an equilateral triangle so that the nearest edges were 1·5 cm. apart. A known antigen was placed in one cup, serum containing an antibody active against this antigen in another cup and the unknown antigen in the third. The antigens and antibody diffused into the gel and a line of precipitate formed where the concentrations were optimal. If the unknown antigen was identified, at least in part, with the known antigen a continuous band of precipitate was found between the two antigen-containing cups and the cup containing the antibody (Fig. 1). This is known as the reaction of identity.

Cold agglutination of red blood cells of protein fractions was determined by setting up Lee and White coagulation tubes in a cold room, adding one drop of a suspension of thrice washed O Rh negative cells, and then 3 drops of one of the eluents from the paper electrophoretogram. The contents of the tubes were mixed thoroughly and allowed to stand for 1 hour at 10° C., when samples from the tubes were examined under a microscope for agglutination.

EXPERIMENTAL RESULTS.

The antibodies present in the Coombs serum were first identified by gel-diffusion by placing the undiluted Coombs serum in one cup of the agar plate, normal serum diluted 1/200 with normal saline in another and eluted γ globulin in the third. Three lines of precipitate formed between the cup containing Coombs serum and that containing the diluted normal serum. One of these lines showed a reaction of identity (Ouchterlony, 1953) with the single line

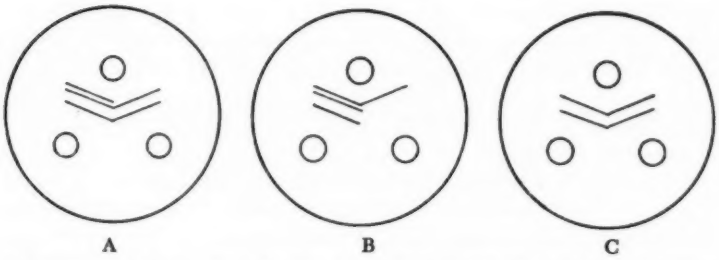

Fig. 2. In each of these figures the cup at the apex contained Coombs serum.

2A The left hand cup contained normal serum and the right hand cup eluted β globulin. Two bands showing reaction of identity and due to β globulins.

2B Left hand cup contained normal serum and the right hand cup eluted γ globulin. One band showing reaction of identity and due to γ globulin.

2C Left hand cup contained cold agglutinins and the right hand cup eluted β globulins. Two bands showing reactions of identity and due to β globulins.

appearing between Coombs serum and γ globulin (Fig. 2B) and the two other lines showed reactions of identity with the two lines appearing between Coombs serum and β globulin (Fig. 2A). Thus the Coombs serum contained at least three antibodies, one anti-γ globulin and two anti-β globulins.

Gel diffusion plates were prepared in which the three cups contained Coombs serum, eluted β globulin and the detached cold agglutinin from the patient's red cells respectively. Two lines formed between the cups containing the Coombs serum and the detached cold agglutinin and these lines showed a reaction of identity with the two lines which appeared between the eluted β globulin and Coombs serum (Fig. 2C).

A control was performed in which normal red cells were treated in exactly the same way as the patient's cells and the resulting eluent was diffused in an Ouchterlony plate with Coombs serum but no lines developed between the cups.

Cold agglutinins were demonstrated in the eluted β globulin fraction of the patient's serum but no agglutination occurred with eluted albumin, α_2 globulin, or γ globulin.

DISCUSSION.

The Coombs anti-globulin serum was demonstrated to have two antibody components which caused precipitation with β globulin isolated from normal serum by paper electrophoresis. The identification of the fractions of β globulin is being investigated and will form the basis of a future report and it is possible that these components will prove to be β_1 and β_2 globulins. Neither antibody component was present in any fraction of normal serum other than the mixed β globulins and, therefore, the technique was adequate for the identification of β globulins.

The eluate from the red cells of a patient with a high titre of cold agglutinins was demonstrated to contain a protein reacting identically with β globulin, both bands of precipitate being present, but the eluate from normal cells contained no such protein. This finding was supported by the demonstration of cold agglutinin activity in only the β globulin fraction of the patient's serum. It is of considerable interest that two bands of precipitate were present but the precise significance of this phenomenon awaits clarification.

The positive identification of the antibody causing the phenomenon of cold agglutination as a β globulin confirms the suggestions of other workers that it differs from iso-immune antibodies in Rh sensitization which are γ globulins.

The gel-diffusion technique described is particularly useful for the identification of antibodies in haemolytic syndromes with a positive Coombs test and some patients with such syndromes are being investigated.

SUMMARY.

A simple technique for the accurate identification of the protein adsorbed to the red cells of patients with acquired haemolytic anaemia has been described. A cold agglutinin adsorbed to the red cells of a patient with acquired haemolytic anaemia was demonstrated to be a β globulin. It is suggested that the method could be applied to the study of the type of protein adsorbed to red cells in other haemolytic states.

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VARIANTS OF *P. HOMINIS*

5. ISOLATION OF THE HEAT-RESISTANT VARIANT.

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In paper 4 of this series it was shown that the inactivation rate of a type II strain of *P. hominis* by heat or formaldehyde indicated the presence of virus unusually resistant to either of these agents (Stanley *et al.*, 1956). Two possibilities were suggested to explain this phenomenon. Either some virus particles were protected from the inactivating agents by aggregation or virus particles inherently resistant to the inactivating agents were present in the preparation. The purpose of this communication is to describe experiments the results of which suggest to us that virus inherently resistant to heat is the more likely explanation.

Materials and Methods.

The same MEF_1 strain of type II poliovirus as previously described (paper 4) was used in this work. Tests for live virus were carried out in tissue cultures of monkey kidney epithelial cells grown in bovine amniotic fluid (Enders). Heat inactivation was carried out at 50° C. in a water-bath of large volume. The virus suspension was dispensed in 1 ml. amounts in thin-walled glass ampoules. Four tissue culture tubes were inoculated with each dilution in estimating the I.D. 50 of infective material.

EXPERIMENTAL.

The elimination of "clumping" as a protection against heat.

The effect of clumping was investigated both by filtration and ultracentrifugation. Infected tissue culture fluids were pooled and centrifuged at 3,200 r.p.m. for 20 minutes. The supernatant fluid was removed and referred to as the "STOCK" suspension. One portion of the "STOCK" suspension was filtered through a Seitz D9 filter pad and the filtered material was referred to as "FIL-

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INACTIVATION OF *P. HOMINIS* BY HEAT (50°C.)

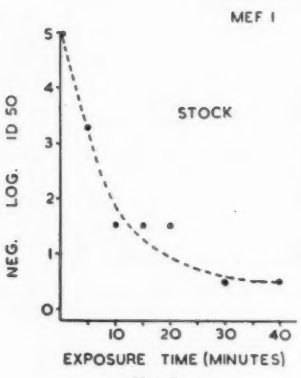

Fig. 1

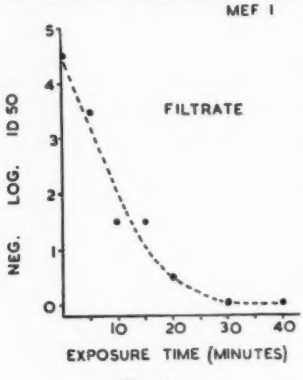

Fig. 2

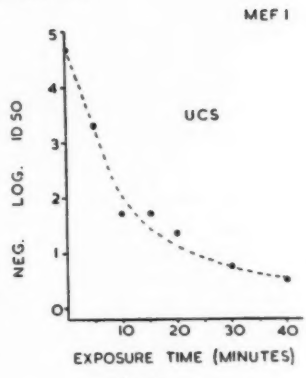

Fig. 3

TRATE". A second portion was subjected to centrifugation at 10,000 r.p.m. for 1 hour in a Spinco preparative Model L centrifuge. The supernatant fluid was recovered and referred to as "U.C.S." Heat inactivation curves were carried out with "STOCK", "FILTRATE" and "U.C.S." suspensions at 50° C. The results (see Figs. 1, 2 and 3) show that there is no significant difference in the inactivation curves of the three suspensions. This suggested to us that "clumping" was unlikely to be responsible for the reduction in inactivation rate. Attempts were then made to isolate virus inherently resistant to heat.

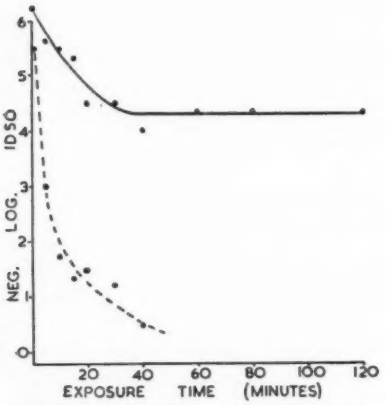

Fig. 4. Inactivation of *P. hominis* by heat (50° C.).

———— Virus recovered after heating at 50° C. for 40 minutes (see text).

— — — Original virus passaged without heat.

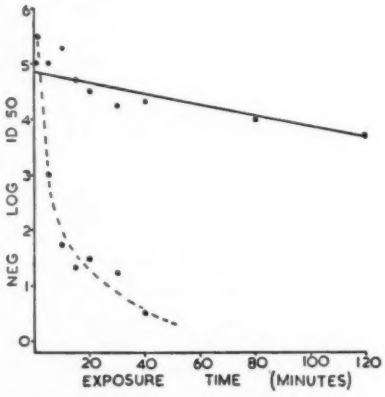

Fig. 5. Inactivation of *P. hominis* by heat (50° C.).

————Heat-resistant virus after 4 passages without heat.

— — —Original virus passaged without heat.

Isolation of virus inherently resistant to heat.

Virus surviving exposure to heat at 50° C. for 40 minutes in the inactivation of the FILTRATE suspension received one passage and was again exposed to heat for 40 minutes. This process was repeated twice and a further heat inactivation test carried out. The curves shown in Fig. 4 indicate that the virus population comprising this passage differs markedly from the original preparation. After four additional tissue culture passages without heat there was no reversion to the curve of the control material (see Fig. 5). Following this exposure to heat at 50° C. for 40 minutes, the virus was exposed twice for 80 minutes, once for 2 hours, and once for 24 hours. The inactivation curve obtained after this treatment is shown in Fig. 6.

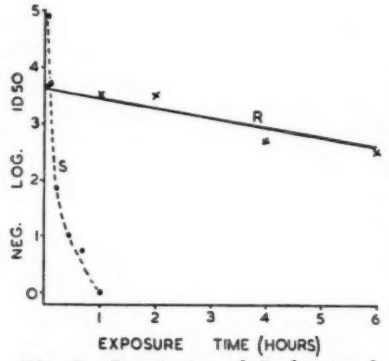

Fig. 6. Inactivation of *P. hominis* by heat (50° C.).

R Heat-resistant virus.

S Original virus passaged without heat.

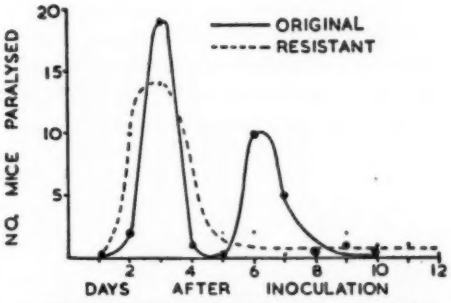

Fig. 7. Results of intracerebral inoculation of 3-week-old mice with (1) heat-resistant virus and (2) original virus suspension.

Properties of the heat-resistant variant.

Intracerebral inoculation of mice revealed that the heat-resistant virus gave only one peak of onset of paralysis compared with the two which normally characterized the original suspension (see Fig. 7).

The original MEF_1 strain has always caused paralysis in monkeys following intracerebral inoculation. The heat-resistant strain did not paralyse a cynomolgus monkey following intracerebral inoculation of 0·5 ml. into the left thalamus.

The resistant variant is not inactivated by exposure at 50° C. for 24 hours, whereas the original strain passaged for the same number of times without heat is inactivated within 1 hour (see Fig. 6).

DISCUSSION.

Curves showing the rate of inactivation of a MEF_1 strain of type II poliovirus have consistently indicated the presence of virus unusually resistant to heat. The failure to alter the shape of this curve by filtration or ultracentrifugation in an attempt to remove clumps suggests that aggregates of virus may not be responsible for the resistance to heat. This suggestion received support when virus particles resistant to heat at 50° C. were selected by subculture from fluids exposed to heat for varying times. The heat-resistant virus has thus far retained its heat resistance when passaged without heating and appears to differ from the heat-sensitive virus in two other respects, viz. (1) it has lower paralysing capacity for monkeys, and (2) it is associated with the first peak of onset of paralysis in mice (see Stanley *et al.*, 1956).

It is thus likely that the shape of the inactivation curve depends on the relative number of heat-sensitive or heat-resistant virus particles present in the virus suspension. If this be true, it is unlikely that all strains of poliovirus would show the same type of inactivation curves. It is not established yet whether a similar explanation may be offered to account for the presence of virus resistant to formaldehyde (Stanley *et al.*, 1956).

SUMMARY.

A variant of type II poliovirus surviving exposure to heat at 50° C. for 24 hours was obtained by subculture of a virus suspension already exposed to heat. The original heat-sensitive strain was inactivated under similar conditions within 1 hour. The heat-resistant variant possessed lower paralysing capacity for monkeys and was associated with the first peak of onset of paralysis in mice.

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THE EFFECT OF TOLUIDINE BLUE ON SERUM LIPIDS AND LIPOPROTEINS IN RABBITS

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The action of heparin on lipid transport is well known. Hahn (1943) observed that heparin *in vivo* reduced visible lipemia following fat ingestion and subsequent work demonstrated that this action was accompanied by a reduction in total fatty acids (Brown, 1952) and in Sf 12-20 lipoproteins (Graham *et al.*, 1951). On the other hand, heparin antagonists have been shown to produce the reverse changes. Protamine increased visible lipemia and total fatty acids during alimentary lipemia (Brown, 1952; Spitzer, 1953) and increased total lipids in the fasting state (Bragdon and Havel, 1954). Toluidine blue increased the Sf 12-20 lipoproteins in fasting rabbits (Hewitt *et al.*, 1953). These increases in serum lipids by heparin antagonists were presumably brought about by their reduction in the heparin clearing effect. Spitzer (1953), however, was unable to produce changes in visible lipemia with toluidine blue and he considered that the fundamental actions of protamine and toluidine blue on heparin differ, the former abolishing both anticoagulant and anti-lipemic effects, the latter only affecting the anticoagulant action.

In view of these observations it was thought worthwhile to investigate the effect of administration of toluidine blue on serum lipids and lipoproteins and in particular to determine whether serum lipids levels could be maintained continuously at a high level by regular administration of toluidine blue. In this paper therefore the effects of daily subcutaneous administration of toluidine blue on the fatty acids, cholesterol, phospholipids and lipoproteins of serum are described, and the possible relationship of these effects to the physiological rôle of heparin in blood lipid transport and to the pathogenesis of atherosclerosis is discussed.

Methods.

In the first series twenty rabbits of mongrel stock of both sexes were used. The rabbits were approx. six months of age and body weights varied from 1·8 to 2·2 kg. These rabbits

[1] This work was aided by a grant from the National Health and Medical Research Council.

were randomised into two groups of ten. The first group received daily injections of 4 ml. saline subcutaneously while the second group received 60 mg. (in 4 ml.) of toluidine blue subcutaneously. All animals were maintained on a normal rabbit mash diet. Total cholesterol, total esterified fatty acids and phospholipids were determined in the serum of each rabbit immediately prior to the commencement of injections and at 7 days and 14 days after commencement. In addition, serum lipoprotein estimations were performed at 7 days and 14 days.

In the second series two groups of four rabbits of the same age, etc. (1·9-2·4 kg.) were treated similarly, one group with saline the other with toluidine blue for a period of 7 days. No further injections were given after 7 days. Blood lipids were determined at 3 days, 7 days and 14 days, i.e. the last determination one week after the cessation of the injections.

Total Cholesterol was determined by the method described by Zlatkis, Zak & Boyle (1953). The volume of the reagents was doubled, 0·1 ml. serum used, and the colour read in a Bausch & Lomb monochromatic photocolorimeter at 550 mu.

Phospholipids. Lipid phosphorus was determined as described by Brown (1954). An aliquot quantity of the extract prepared for fatty acid determination was used and the method modified to a final volume of 10 ml. The colour was read in a "Unicam" S.P. 600 spectrophotometer at 820 mu and the lipid phosphorus thus determined was converted to phospholipid by multiplying by a factor of 25.

Fatty acids. Total esterified fatty acids were determined as described by Stern & Shapiro (1953). Dispersion of serum was aided by the use of a pipette and vibrator as described by Brown (1954) and the colour was read in a "Unicam" spectrophotometer at 520 mu.

Lipoproteins. The lipoproteins were determined by paper electrophoresis according to the method of Swahn (1953). The paper strips were divided into 3 sections corresponding to α_1 globulin (α-lipoprotein) to β globulin (β-lipoprotein) and the remainder of the strip to the point of origin (mainly neutral fat). These 3 sections were eluted and the results expressed quantitatively as mg. triolein/100 ml. serum.

RESULTS.

Following administration of toluidine blue all lipid fractions of the serum were elevated. All results are expressed as mg./100 ml. serum with the standard deviation following. In the first series of rabbits the total fatty acids increased from a pre-injection level of 193 (83) mg. p.c. to 722 (166) mg. p.c. in 7 days and 746 (182) mg. p.c. in 14 days. In the control group a small rise from 177 (47) mg. p.c. to 225 (40) mg. p.c. at 7 days and 260 (79) mg. p.c. at 14 days was recorded. Fig. 1 shows these changes represented graphically where the mean total fatty acid levels of the first series of rabbits are plotted against the time in both the control and toluidine blue groups.

In the case of the cholesterol and phospholipids similar changes but of a lesser degree were shown. Thus the cholesterol rose from a pre-injection level of 148 (27) mg. p.c. to 182 (23) mg. p.c. and 184 (30) mg. p.c. at 7 days and 14 days respectively in the toluidine blue group while the corresponding values in the control group were 132 (24) mg. p.c., 111 (28) mg. p.c. and 108 (29) mg.

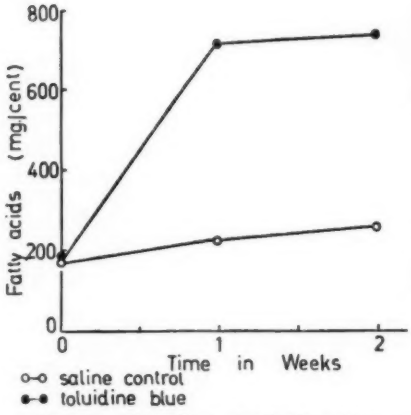

Fig. 1. The effect of toluidine blue on total fatty acids of rabbits. Details in text.

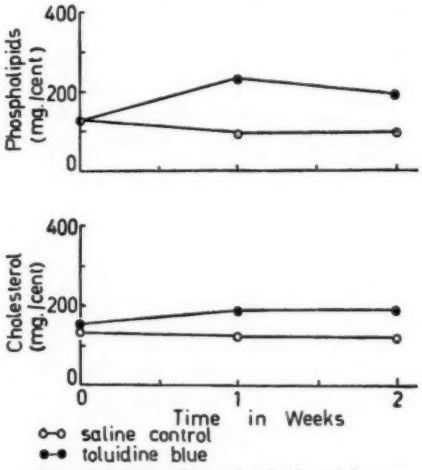

Fig. 2. The effect of toluidine blue on cholesterol and phospholipids of rabbits. Details in text.

p.c. Phospholipids rose from 118 (30) mg. p.c. to 216 (24) mg. p.c. and 195 (55) mg. p.c. at 7 days and 14 days respectively, whereas in the control series the level remained more or less constant at 116 (18) mg. p.c., 96 (23) mg. p.c. and 99 (24) mg. p.c. for the corresponding times. Fig. 2 shows the changes in cholesterol and phospholipid following toluidine blue administration compared with the changes in the control group over the same period.

The elevations in all 3 fractions have been shown to be highly significant statistically.

It will be noted from Figs. 1 and 2 that the changes are fixed at 7 days, no further appreciable elevation in any fraction occurring in the succeeding 7 days.

TABLE 1.

Serum lipoproteins following toluidine blue administration.

		7 days	14 days
Control (Mean of 10 rabbits)	Neutral Fat	150(14)*	106(44)
	β-lipoprotein	137(29)	148(58)
	α-lipoprotein	115(15)	132(99)
	Total fat	402(66)	386(146)
Toluidine blue (Mean of 10 rabbits)	Neutral Fat	328(111)	233(91)
	β-lipoprotein	405(113)	586(110)
	α-lipoprotein	150(58)	120(85)
	Total Fat	883(177)	903(198)

*Standard deviation.

It would appear therefore that the change has become static during this period, the plasma lipid levels being set at a higher level by the toluidine blue and maintained at this level while its administration is continued.

Table 1 shows the changes in serum lipoproteins following toluidine blue administration. The estimations were not performed prior to commencement of the experiment, but only at 7 days and 14 days after injections were started. It can be seen that the elevation in lipoprotein produced by toluidine blue is borne almost entirely by the β-lipoprotein. Thus at 7 days the β-lipoprotein in the control series is 137 (29) mg. p.c., whereas in the toluidine blue group this has been elevated to 405 (113) mg. p.c. At 14 days the β-lipoprotein has been elevated from 148 (58) mg. p.c. in the control series to 586 (110) mg. p.c. in the toluidine blue group. These changes in β-lipoprotein are highly significant.

The corresponding changes in the α-lipoprotein are practically nil, 115 (15) mg. p.c. in the control group and 150 (58) mg. p.c. in the toluidine blue group at 7 days, and 132 (99) mg. p.c. and 120 (85) mg. p.c. respectively at 14 days.

TABLE 2.

Serum lipids following toluidine blue and subsequent withdrawal.

		0 days	3 days	7 days	14 days
Control (Mean of 4 rabbits)	Cholesterol	121(20)*	118(28)	138(26)	141(22)
	Phospholipid	142(20)	123(20)	153(22)	129(13)
	Fatty acids	219(52)	170(27)	165(54)	253(21)
Toluidine Blue (Mean of 4 rabbits)	Cholesterol	107(6)	151(12)	182(14)	157(47)
	Phospholipid	132(46)	177(10)	283(24)	172(63)
	Fatty acids	275(88)	412(23)	810(24)	420(204)

*Standard deviation.

Table 2 shows changes in the serum lipids in the second series of rabbits. In this group recovery has been allowed to occur in order to demonstrate the temporary nature of the lipid changes and to show that return to normal lipid levels occurs following withdrawal of toluidine blue. It can be seen that similar elevations of serum lipids occur in the first 7 days to those recorded in the first series. In this series estimation of the levels at 3 days shows that the changes are well established but not maximal at this time. At 14 days, i.e. 7 days after the withdrawal of the toluidine blue, the values of all fractions have been considerably reduced, practically to the control level. One animal remained at a somewhat higher level, however, and this has disproportionately increased the average values reported. It is noteworthy that this animal had during this time developed a widespread infection of an injection-site and possibly this was associated with slow excretion of toluidine blue (in this animal) and consequent slow return to normal. In all the other animals, however, the levels had fallen to pre-injection levels.

DISCUSSION.

Toluidine blue has been shown to produce marked changes in serum lipids and serum lipoproteins. These changes can be explained most satisfactorily by assuming that heparin is physiologically concerned with lipid transport and that toluidine blue interferes with this lipid transport function. A comparison of the effects of toluidine blue and of heparin on the serum lipids and lipoproteins is of interest. Thus heparin has been shown to reduce the total fatty acids (Brown, 1952), reduce the cholesterol (Basu & Stewart, 1950), reduce the β-lipoprotein (Nikkila, 1953) and the Sf 12-20 lipoprotein (Graham *et al.*, 1951). All of these changes can be reversed by administration of toluidine blue. Thus we have demonstrated that toluidine blue elevates the total fatty acids, cholesterol and phospholipid and is associated with an increase of β-lipoprotein. These changes suggest that toluidine blue does antagonise the lipid transport effects of heparin, and in this property does not differ fundamentally from protamine as was suggested by Spitzer (1953) in his work with dogs. The rabbit is perhaps a poor choice of animal on which to demonstrate the effects of heparin and heparin antagonists, since some controversy exists as to the effect of heparin on lipemia clearing in the rabbit. Thus Nikkila (1953) has been unable to show any reduction in visible lipemia following heparin administration. Comfort (1955), however, has shown that changes in lipoprotein may occur following heparin administration independent of changes in visible lipemia, and Graham *et al.* (1951) have certainly demonstrated with rabbits the effect of heparin in reducing the Sf 12-20 lipoproteins. Our work reported here suggests, too, that in the rabbit, heparin is not only present, but is active in removing circulating lipids from the blood, an action which can be blocked by toluidine blue. Whether or not circulating heparin is affected by toluidine blue is a moot point. It is conceivable that heparin is liberated according to a physiological stimulus, and that toluidine blue antagonises it, not so much in the circulation, but at its site of formation, the tissue mast cell. It is well known that toluidine blue is taken up by the mast cell, and possibly this action antagonises heparin at its source, preventing its liberation and consequent effect on lipid removal, and thus allowing lipid to accumulate in the blood. The net result on lipid transport, however, would be the same, i.e. removal of heparin and consequently higher lipid levels.

These observations lend considerable weight to the possibility already conceded by other workers that the rôle of heparin in the body is not that of a circulating anticoagulant but a rôle concerned with the removal of lipid from the blood. This action of heparin is possibly associated with transfer of lipid from the low density to the high density lipoproteins (Graham *et al.*, 1951) or with lipolysis and removal in this way (Grossman *et al.*, 1955). It has been shown that the amount of heparin required to produce anticoagulant effects is some 10 times that required to produce anti-lipemic effects (Spitzer, 1954). The amount

of heparin present in the circulating blood is only 9 micrograms/100 ml. (Jaques, 1949), this amount being insufficient to produce anticoagulant effects but sufficient to produce lipemia clearing effects. Hartmann & Meng (1955) have shown, however, that in "platelet free" plasma, the effects of heparin on clotting are much more marked, and may correspond more to the order required to produce anti-lipemic changes. However, the fact still remains that in the physiological situation, there is not sufficient heparin in the blood to produce anticoagulant effects, whereas there is quite sufficient to produce anti-lipemic effects. It is therefore fair to conclude that heparin is important as a physiological lipid transport mechanism.

The possibility exists, however, regarding the action of toluidine blue, that it produces its changes in lipids independent of any heparin antagonist effect. Two other possible mechanisms exist. Toluidine blue is a toxic substance (Leitch & Haley, 1952) and it is possible that cellulo-toxic changes occur with resultant fat hold-up. If these changes are present they are certainly not permanent nor marked since a rapid fall in serum lipids occurs following withdrawal of toluidine blue, i.e. once the dye is excreted the chemical effect is removed and serum lipids return to normal. The other possibility is that toluidine blue acts by virtue of adrenal stimulation — the stress response — with liberation of cortisone, and consequent cortisone effects on lipids. Cortisone produces elevation of all lipid fractions in rabbits (Wang *et al.*, 1955) and the elevations recorded by these workers are of the same magnitude as those recorded here.

One further point remains to be considered briefly. It is well substantiated that atherosclerosis is associated with an elevation of serum lipids (Keys, 1951; Gofman *et al.*, 1950; Barr, 1953) and heparin has been shown also to be associated with the control of these lipids (Weld, 1944; Basu & Stewart, 1950; Graham, 1951, etc.). In this work the continual removal of heparin results in a considerable and sustained elevation in plasma lipids. If we concede that the action of heparin on lipid transport is a physiological one, and the evidence certainly supports this contention, then the absence of this mechanism would result in an elevation of blood lipids as has been demonstrated here. It is possible, therefore, that the chronic elevation of lipids which would result from spontaneous failure of the heparin clearance mechanism may be a feature in the pathogenesis of atherosclerosis. This aspect of the problem is being further investigated in this laboratory and will be the subject of a further communication.

SUMMARY.

Daily subcutaneous administration of toluidine blue has been shown to elevate significantly the total serum fatty acids in rabbits. An elevation of some 300 p.c. was observed.

These changes were accompanied by elevations in the cholesterol, phospholipids and in the β-lipoproteins. No changes occurred in the α-lipoproteins.

The elevations were maintained for as long as the toluidine blue was administered but fell following its withdrawal.

The action of toluidine blue was discussed in terms of the physiological rôle of heparin in lipid transport and the possible relationship of this to the pathogenesis of atherosclerosis.

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SEVERITY OF ATHEROSCLEROSIS IN RABBITS IN RELATION TO SERUM LIPIDS AND TO AORTA CHOLESTEROL CONTENT

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Various workers have shown an elevation in serum lipids and lipoproteins associated with atherosclerosis in man. High cholesterol, high fatty acids, increased cholesterol/phospholipid ratio and elevation in lipoprotein fractions have all been shown to be associated with the presence of atheroma (Gertler, Garn and Lerman, 1950; Gofman *et al.*, 1950; Barr, Russ and Eder, 1951; Nikkila, 1953). At the moment considerable controversy exists as to which factor represents the best "atherogenetic index". Meng & Davis (1955) have demonstrated a correlation between the serum cholesterol in cholesterol fed rabbits and the severity of the resulting atheroma. There has been, however, relatively little attempt to correlate the other serum lipid and lipoprotein levels in cholesterol fed animals with the severity of the atheroma produced. It was thought that such data would shed some light on the relative importance of the various lipid fractions in atherogenesis.

In this paper the severity of atheroma in cholesterol fed rabbits is related to the serum levels of cholesterol, phospholipid, total fatty acids and of α- and β-lipoproteins in an attempt to show which serum lipid fraction corresponds most closely to the degree of atheroma.

Further, since McMillan, Horlick & Duff (1955) have failed to show a close relationship of aorta cholesterol with macroscopic grading in experimental atheroma, the present work was extended and the cholesterol content of the aortae determined and related to the degree of atheroma shown by the rabbits.

METHODS.

In the first series, thirteen rabbits of mongrel stock approx. 6 months of age were used. The sexes were mixed and the animals varied in weight from 1·38 to 1·96 kg. All of these animals received daily 75 gm. of rabbit mash containing 0·75 gm. cholesterol and 4·5 ml. peanut oil. This diet was continued for 8 weeks. Cholesterol, phospholipid, total esterified fatty acids and lipoproteins were determined in the serum at 4, 6 and 8 weeks after the commencement of the cholesterol feeding. At 8 weeks the rabbits were sacrificed and their aortae

removed. The degree of atheroma present was graded macroscopically and the cholesterol and phospholipid contents of the aorta were determined chemically.

In the second series nine rabbits of mongrel stock (1·52-1·84 kg.) were used. These animals were receiving "Ovadol" (a fat soluble vitamin supplement emulsion) until 1 week before commencement of the experiment. They were fed on a diet consisting of 75 gm. rabbit mash with 1 gm. of cholesterol and 6 ml. peanut oil daily. This was continued for 8 weeks. Serum lipids were determined at 4 and 8 weeks and the animals were sacrificed at 8 weeks, their aortae removed, the atheroma graded, and the cholesterol and phospholipid content of the aorta determined.

Serum Lipids. Serum cholesterol was determined by the method of Zlatkis, Zak & Boyle (1953), phospholipid by the method of Brown (1954) and total esterified fatty acids as described by Stern & Shapiro (1953).

Serum Lipoproteins were determined by paper electrophoresis by the method described by Swahn (1953).

The slight modifications adopted in this laboratory for these lipid and lipoprotein determinations have been described elsewhere (Day, Wilkinson & Schwartz, 1956).

Grading of Atheroma. The severity of atheroma was assessed by macroscopic grading of the fresh unstained aorta observing the artery in its whole extent from the root of the aorta to its bifurcation into the common iliacs. The atheroma was graded into five grades of severity (I-V). This arbitrary assessment was made on the basis of the number of lesions present, their size and extent, the total surface area of the aorta involved and the amount of "heaping up" which occurred in the more extensively involved arteries.

Grade I. Minimal but definite atheromatous lesions involving no more than 5-10 p.c. of surface area.

Grade II. More than a dozen discrete or confluent lesions involving 10-20 p.c. of the surface area.

Grade III. More confluent lesions with some definite "heaping up" in the aortic arch and involving a surface area of 20-40 p.c. of the total.

Grade IV. Extensive confluent lesions involving 40-60 p.c. of the surface area.

Grade V. Gross involvement of the aorta in its whole extent with much heaping up of the lesions and a surface area involvement of over 60 p.c. of the total.

The lesions were all graded by the same person who was ignorant of the source of the artery.

Aorta Cholesterol. The aorta was removed *in toto* from its root to its bifurcation and cleaned meticulously of all extraneous connective tissue. Its wet weight was recorded. It was cut into small pieces approximately 1 mm. × 3 mm. and extracted for 6 hours with equal parts of chloroform: methanol in a Soxhlet continuous extraction apparatus. The residue was allowed to stand overnight in ether and the 2 extracts pooled, evaporated to dryness in a water bath below 70° C. and taken up with warm petroleum ether to a final volume of 100 ml. An aliquot quantity of this extract was evaporated to dryness in a water bath below 70° C. and the cholesterol in the residue taken up in glacial acetic acid and determined as for serum cholesterol. The aorta cholesterol was expressed as mg. per aorta.

Aorta Phospholipid. Estimation of aorta phospholipid was made on the extract prepared for aorta cholesterol determination. An aliquot quantity of this extract was evaporated to dryness below 70° C. in a water bath and aorta lipid phosphorus determined as for serum lipid phosphorus. The volumes were expressed as mg. phospholipid per aorta.

RESULTS.

Table 1 shows the lipid data for the first series of thirteen rabbits. The aorta cholesterol and phospholipid are expressed as mg. per aorta. The serum lipids and lipoproteins are the mean of the 4, 6 and 8 weeks determinations and are expressed as mg. per 100 ml. of serum. Table 2 shows the correlation coefficients of this same group of animals set out as a correlation matrix for the various lipid fractions. It can be seen from Tables 1 and 2 that all the serum lipid fractions (except the cholesterol/phospholipid, C/P, ratio) are highly correlated with each other and as a consequence all are highly correlated with the atheroma grading. Fig. 1 shows the correlation of serum cholesterol with the aorta grading. In the serum lipoproteins there is no appreciable correlation of α-lipoprotein either with the other serum fractions or with atheroma grading. There is, however, a close correlation between β-lipoproteins and atheroma grading. It should be noted that the β-lipoprotein was determined by paper electrophoresis and as the division of the paper into the neutral fat band (possibly including a β-lipoprotein trail) and the β-lipoprotein band was rather arbitrary and as the α-lipoprotein showed no appreciable changes, the total fat estimated was included in the matrix since it virtually corresponds to β-lipoprotein, but is subject to smaller errors of estimation.

It can also be seen from Tables 1 and 2 that a very high correlation exists between the aorta grading and aorta cholesterol (correlation coefficient = 0·96).

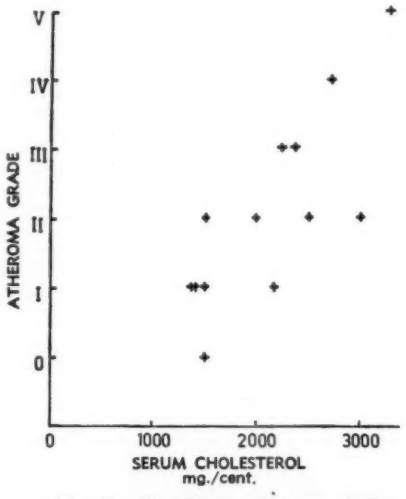

Fig. 1. Correlation of serum cholesterol level with severity of atheroma in cholesterol fed rabbits. Details in text.

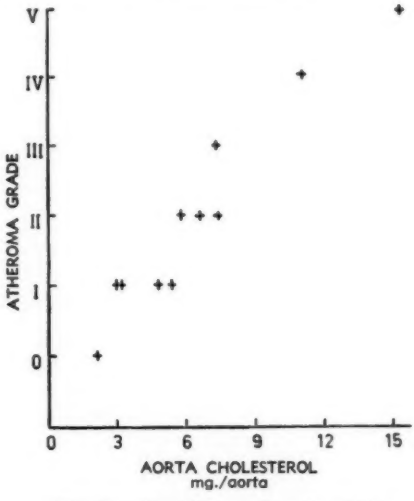

Fig. 2. Correlation of aorta cholesterol content with macroscopic grading of atheroma in cholesterol fed rabbits. Details in text.

TABLE 1.

Aorta and serum lipid data of first series of cholesterol fed rabbits.

Atheroma	Aorta Cholesterol	Aorta Phospholipid	Serum cholesterol	Serum phospholipid	Serum C/P ratio	Serum fatty acids	Neutral Fat	β-lipoprotein	α-lipoprotein	Total fat
0	2·2	1·6	1537	584	2·71	910	550	1900	150	2570
I	4·8	2·2	1497	599	2·53	827	445	1670	130	2220
I	5·4	2·7	2170	918	2·41	1493	590	3090	210	3900
I	3·2	1·8	1410	671	2·09	1127	460	1730	160	2350
I	3·0	1·7	1410	447	3·19	683	450	1450	160	2060
II	6·6	2·2	3003	1066	2·79	1667	750	3570	150	4480
II	7·4	3·1	2553	1031	2·47	1600	770	3400	130	4300
II	5·8	1·9	1550	659	2·37	983	670	2140	170	2970
II	—	—	1970	898	2·16	1207	370	2070	180	2530
III	—	—	2387	1054	2·22	1583	350	3220	130	3690
III	7·4	3·5	2253	821	2·67	1130	690	2970	180	3840
IV	11·0	1·8	2770	1052	2·59	1480	600	3600	170	4360
V	15·4	6·7	3293	1293	2·51	2067	730	3880	160	4770

TABLE 2.

Correlation matrix of first series of cholesterol fed rabbits.

		Aorta		Serum					
		Atheroma	Cholesterol	Cholesterol	Phospholipid	Fatty acid	β-lipoprotein	Total fat	C/P ratio
Aorta	Atheroma grade	1·00	0·96	0·79	0·85	0·71	0·75	0·72	−0·11
	Aorta cholesterol		1·00	0·85	0·86	0·80	0·81	0·80	−0·12
Serum	Cholesterol			1·00	0·95	0·92	0·96	0·94	0·02
	Phospholipids				1·00	0·98	0·93	0·90	−0·29
	Fatty acids					1·00	0·92	0·89	−0·27
	β-lipoprotein						1·00	0·99	−0·06
	Total fat							1·00	−0·01
	C/P ratio								1·00

(5 p.c. significance level one tailed test $r = 0·44$)

TABLE 3.

Aorta and serum lipid data of second series of cholesterol fed rabbits.

Atheroma	Aorta Cholesterol	Aorta Phospholipid	Serum Cholesterol	Serum Phospholipid	Serum C/P ratio	Serum Fatty acids	Neutral fat	β-lipoprotein	α-lipoprotein	Total fat
I	4·7	4·4	1980	868	2·28	1255	960	2680	160	3800
I	3·0	2·5	2815	1010	2·73	1670	720	1720	220	2440
I	4·7	3·3	1905	705	2·75	995	730	1880	160	2770
III	9·3	4·4	3595	1125	3·20	1615	1520	3280	170	4970
III	8·3	4·8	2700	1038	2·62	1585	1420	2320	160	3900
III	8·5	5·0	3140	965	3·70	1355	420	3040	150	3610
IV	10·5	5·3	2820	843	3·30	1610	1200	2440	110	3750
IV	9·8	4·6	3230	1025	3·16	1775	1580	3400	110	5090
IV	12·5	6·1	3000	833	3·64	1375	1520	3120	200	4840

TABLE 4.

Correlation matrix of second series of cholesterol fed rabbits.

		Aorta		Serum					
		Atheroma	Cholesterol	Cholesterol	Phospholipid	Fatty acid	β-lipoprotein	Total fat	C/P ratio
Aorta	Atheroma grade	1·00	0·96	0·69	0·24	0·51	0·68	0·75	0·80
	Aorta cholesterol		1·00	0·61	0·11	0·31	0·72	0·80	0·83
Serum	Cholesterol			1·00	0·74	0·74	0·64	0·61	0·69
	Phospholipid				1·00	0·77	0·38	0·38	0·03
	Fatty acids					1·00	0·28	0·39	0·26
	β-lipoprotein						1·00	0·92	0·55
	Total fat							1·00	0·53
	C/P ratio								1·00

(5 p.c. significance level one tailed test $r = 0·52$)

Fig. 2 shows this relationship. The cholesterol: phospholipid ratio is not correlated with atheroma grading or with the other fractions. The small negative correlations in the Table are due to the high ratio at 8 weeks of one rabbit. In this case the cholesterol fraction was relatively higher than the other fractions.

Table 3 gives the lipid data, etc., for the second series of rabbits. Again aorta lipids are expressed as mg. per aorta. The serum lipids are the mean of the 4 and 8 weeks determinations and in the lipoproteins only the 4-week determinations were available. Table 4 shows the correlation matrix of this second series.

It can be seen from Tables 3 and 4 that cholesterol is correlated with all fractions including the C/P ratio and is correlated with the degree of atheroma developed. The phospholipid and fatty acid fractions which are correlated with each other are only poorly correlated, if at all, with β-lipoprotein (and the virtually equivalent measurement total fat) and with the aorta cholesterol. However, the fatty acid fraction does show some correlation with the atheroma grade. The β-lipoprotein, which is correlated with the serum cholesterol, shows good correlation with the atheroma grade. The C/P ratio is also well correlated with the degree of atheroma.

DISCUSSION.

Severity of atheroma has been shown to correlate quite closely with the serum cholesterol. This is perhaps hardly surprising in view of the fact that the animals were cholesterol fed, but it does serve to amplify the fact that the serum level is of vital importance in determining the degree of atheroma. It is worth noting that the levels of cholesterol in the liver (which were also studied in this work but not reported) showed no correlation at all to the atheroma. The precise mechanism of cholesterol deposition in arteries remains obscure, but it has been suggested (Page, 1954) that filtering of blood lipid occurs into the artery and deposition follows the failure of the artery to deal with this filtrate. Thus elevated blood cholesterol would result in an added strain being put on the blood vessel and deposition would result. If this theory were true then we would expect the amount deposited to be directly related to the amount in the filtrate which in turn would be proportional to the serum level.

Similar considerations would apply to the correlation of atheroma with β-lipoprotein. In cholesterol fed rabbits most of the cholesterol is transported in combination as β-lipoprotein and the cholesterol carried in this way has been considered more atherogenetic (Barr *et al.*, 1951). The β-lipoprotein level in rabbits in this present experiment did bear a close correlation to atheroma, but no better than the cholesterol itself, although it must be admitted that the same relationship would be expected. (The cholesterol in the blood of cholesterol fed rabbits is carried almost exclusively as β-lipoprotein.)

The correlation of atheroma with fatty acids and phospholipid varies with the two experiments, close correlation exists in the first series between phospholipids and fatty acids and the serum cholesterol and hence with the atheroma. In the second experiment the rise in cholesterol level was greater (due to the greater amount of cholesterol being fed) and hence the fatty acid and phospholipid levels did not increase at the same rapid rate. Therefore, although the close correlation of serum cholesterol and atheroma is maintained in this second experiment, the correlations between phospholipids and fatty acids with atheroma are very greatly weakened. It would appear that the determining factor is therefore the serum cholesterol level and where other lipid fractions correlate with this they also correlate with the severity of atheroma. However, where other lipid fractions fail to correlate closely with the serum cholesterol this latter factor determines the atheroma and correlates with its severity.

With regard to the different correlation of phospholipid in the two experiments, one very significant point emerges. In the first series there is no correlation at all between the cholesterol/phospholipid (C/P) ratio and the atheroma, whereas in the second series there is a close correlation between the C/P ratio and the severity of atheroma. In the first series the cholesterol rose more slowly allowing the phospholipid to increase proportionately and therefore the C/P ratio did not alter appreciably with rising cholesterol. Thus no correlation existed between the serum cholesterol and the C/P ratio in this series and consequently no correlation was apparent between the C/P ratio and the severity of atheroma. The fact that a correlation between C/P ratio and atheroma exists in the second series is probably due to the fact that in these rabbits serum cholesterol increased at a much greater rate than the serum phospholipid and therefore the C/P ratio was highly correlated with the serum cholesterol and because of this fact was highly correlated with the atheroma grading. It would appear therefore that the important factor which determines the severity of atheroma is the serum cholesterol level (or rather the cholesterol in the β-lipoprotein) and not the C/P ratio. Much interest has centred around the apparent stabilizing effect of the phospholipid on cholesterol and an elevated C/P ratio has been considered by some to be a better "atherogenetic index" than cholesterol level alone (Ahrens & Kunkel, 1949; Gertler *et al.*, 1950). This stabilising effect of phospholipid was particularly supported by the effect of the detergents, e.g. Triton, Tween, etc., in reducing atherosclerosis in cholesterol fed rabbits, presumably by elevating the phospholipid and reducing the C/P ratio (Ladd, Kellner and Correll, 1949; Payne and Duff, 1951). It would be expected, if this were so, that an even closer correlation would exist between the severity of atheroma and the C/P ratio than with the cholesterol and this is certainly not the case in our rabbits. It would appear that in our experiment at least the cholesterol level (or rather that in the β-lipoprotein) and not the C/P ratio determined the level of atheroma. Friedman *et al.* (1955) have

pointed out that the detergents which produce hyper-cholesterolemia and also elevate phospholipid fail to cause a corresponding elevation in the microscopically visible chylomicra and thus the protection credited to increased phospholipid may in fact be due to relatively reduced chylomicron counts.

The very close correlation shown between aorta cholesterol and macroscopic grading is perhaps what could be expected. The lesions consist mostly of cholesterol and therefore, if careful grading occurs taking into account all factors, the chemical estimations must mirror this grading. Possibly the reason why such a close correlation did not exist in the work described by McMillan, Horlick and Duff (1955) was because of the varied amount of heaping up occurring in the aorta with very advanced atheroma. The weight of cholesterol present in the arteries cannot then be taken into account by considering surface area involvement only and ignoring this heaping up. In the work of McMillan *et al.* the rabbits were maintained on cholesterol for 90 days and the cholesterol content of most of the arteries was considerably in excess of that in our work. The error in these grossly atheromatous animals would be greatly magnified by the varying thickness of lesions which would occur more markedly at this stage. It is significant, in this connection, that in Grades 1 and 2 of these workers a much closer correlation exists and the animals showing these grades correspond more closely in aortic cholesterol content to the animals described in our work. We maintain, therefore, that essentially the same information can be obtained from careful macroscopic grading of arteries as from chemical cholesterol determinations, provided the atheroma is not too gross. With advanced lesions, chemical determination may give a more accurate picture because of its objective approach.

SUMMARY.

A close correlation was shown to exist between the severity of atheroma resulting from cholesterol feeding in rabbits and the serum cholesterol and β-lipoprotein levels.

No consistent correlation was shown to exist between the phospolipids, fatty acids and the cholesterol-phospholipid ratio and the severity of atheroma.

There was no correlation between α-lipoprotein and atheroma.

The aorta cholesterol content in the cholesterol fed rabbits was very closely related to the severity of atheroma as determined by macroscopic grading.

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THE PRESERVATION OF COAGULATION FACTORS IN HUMAN PLASMA[1]

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Blood or plasma to which the usual anticoagulants are added and stored at room temperature shows reduced coagulability in a few days due to loss of alpha-prothromboplastin (antihaemophilic factor) and prothrombin accelerator (Factor V, labile factor, plasma Ac-globulin, proaccelerin). A prolongation of the thrombin clotting time is also noted. The mechanisms which are responsible for these changes in stored plasma are not well understood. Since they are of theoretical interest and of practical significance an investigation of the impaired coagulability of stored plasma was carried out and the presevation of the coagulation factors was attempted.

Materials and Methods.

Blood was collected from normal donors with the addition of 1/9th. volume of 0·1M sodium oxalate or 0·13M trisodium citrate. In some cases merthiolate was added to give a final concentration of 1 part in 10^4. After centrifugation, cell free plasma samples were kept for one month at 18-20° C. in soft glass test tubes plugged with cotton wool. Such specimens will be referred to as "stored" plasma. All operations were carried out under sterile conditions. In cases where the plasma was kept in another atmosphere than air the specimens were sealed in pyrex tubes. The control specimens were kept at − 15° C.

Thrombin preparations used were one of bovine origin (Parke-Davis, Thrombin Topical) and the other was isolated from human plasma. Thrombin was first prepared from a portion of the euglobulin fraction of the plasma according to Fantl and Ebbels (1953). To this thrombin solution was added an equal volume of 0·001M sodium oxalate. The fibrinogen was removed from the bulk of the preparation by the addition of one fifteenth of its volume of this thrombin preparation. One twenty-fifth of the volume of 0·025M calcium chloride was then added. The reason for the preliminary defibrination before the bulk of thrombin is prepared is because loss of thrombin by adsorption onto fibrin is avoided and therefore an increased thrombin yield is obtained.

Reagents and details for the determination of thrombin and the relationship between clotting time and thrombin units are given by Fantl (1954a). Antithrombic activity of plasma was measured by the rate of disappearance of added thrombin.

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[1] Part of the expenses of this investigation were defrayed by a grant from the National Health and Medical Research Council.

The pH of the plasma was adjusted by the addition in the cold of N/10 hydrochloric acid and by the use of veronal buffers of Michaelis (1930) or "tris buffer" (trishydroxymethylaminomethane).

Fibrinogen fractions were isolated from plasmas by ether precipitation (Kekwick *et al.*, 1955), or by repeated precipitation with ammonium sulphate to 25 p.c. saturation. The fibrinogen was dissolved in a solution containing 0·12M sodium chloride and 0·026M trisodium citrate and dialysed against a similar solution at 6° C. for 15 hours.

Fibrinogen was determined in 1 ml. plasma aliquots diluted 10 times with 0·15M sodium chloride and clotted by the addition of 0·5 ml. of a potent thrombin solution. After standing one hour the fluid was expressed and the fibrin washed with saline and distilled water. Fibrin-N was estimated by a micro-kjeldahl method (Miller and Houghton, 1945). Dried human ash-free fibrin contained 16·9 p.c. nitrogen.

Hexosamine was determined by the procedure of Schloss (1951) and amide nitrogen was estimated as the ammonia formed by 12 hours hydrolysis with 2 N hydrochloric acid at 100° C. (Leach and Parkhill, 1955).

Prothrombin activity by the one stage technique was carried out according to Quick (1938). Prothrombin was assayed by the method of Fantl (1954a). The rate of formation of thrombin was determined according to Fantl (1954b).

Prothrombin accelerator activity was determined by the technique of Quick and Stefanini (1948), and also by the accelerating effect of the sample on thrombin formation from prothrombin isolated from "stored" plasma according to Fantl and Nance (1948). Elution of the barium sulphate adsorbate was with one half of the original plasma volume of a solution containing 0·043M trisodium citrate and 0·1M sodium chloride.

The activity of alpha-prothromboplastin (antihaemophilic factor) was estimated from the rate of formation and yield of thrombin, either in the plasma itself or in mixtures of stored plasma with plasma from alpha-prothromboplastin deficient patients (alpha-haemophilia). In each case platelets or brain lipids (Bell and Alton, 1954) were added. The assay procedure of Fantl (1954b) was used.

The thromboplastin generation test of Biggs and Douglas (1953) was carried out in citrated plasma. Activities are expressed in terms of normal plasma controls.

RESULTS.

Alteration of Fibrinogen Reactivity during Storage.

It has been observed by Banfi, Tanturi and Bay (1945) that a larger amount of thrombin was required to clot stored plasma than fresh plasma. This was thought to be due to an increase of factors which destroy thrombin (Stefanini, 1948). Therefore, determinations of the antithrombic activity of fresh and stored plasmas were carried out and compared with plasma obtained from a patient suffering from pancreatitis in which condition antithrombin is sometimes increased according to Dreiling *et al.* (1954).

It is apparent from figure 1 that the patient's fresh plasma showed a slightly increased antithrombic activity, but the antithrombic activity of fresh and "stored" oxalated plasma are identical within the experimental errors of the technique employed. The delayed thrombin fibrinogen reaction in stored plasma is therefore not due to an increase of thrombin inactivating factors.

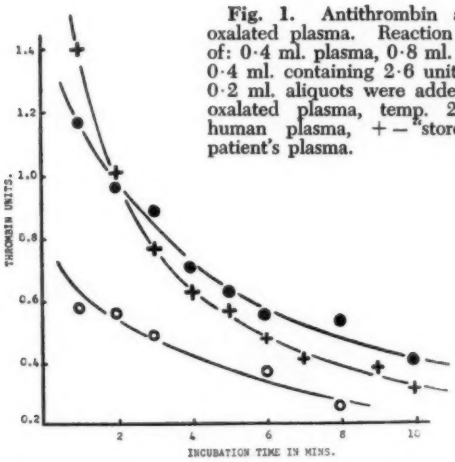

Fig. 1. Antithrombin activity of human oxalated plasma. Reaction mixtures consisted of: 0·4 ml. plasma, 0·8 ml. tris buffer pH 7·2, 0·4 ml. containing 2·6 units bovine thrombin. 0·2 ml. aliquots were added to 0·4 ml. fresh oxalated plasma, temp. 28° C. ● – fresh human plasma, + – "stored" plasma, ○ – patient's plasma.

Nilsson and Wenckert (1954) claim that a normally occurring heparin-like substance is found in higher concentration in stored plasma. "Stored" plasma was tested for heparin as shown in Table 1.

TABLE 1.

Influence of toluidine blue or $BaSO_4$ on the thrombin clotting times of fresh heparinized and "stored" oxalated plasma.

Additions	Thrombin Clotting Time of Plasma (in seconds)		
	Fresh	*Heparinized	Stored
1. 0·15 M NaCl	6½	>60	>60
2. Toluidine blue	7½	7½	>60
3. $BaSO_4$	7	7	>60

1 ml. $BaSO_4$ suspension (230 mg. $BaSO_4$) used for 5 ml. of plasma, 6° C, ½ hour. *Heparinized oxalated plasma contained 5 μg. heparin/ml. Reaction mixtures consisted of: 0·2 ml. plasma plus 0·1 ml. 0·15 M-NaCl or 0·1 ml. 0·15 M-NaCl containing 50 mg. p.c. toluidine blue. 0·1 ml. human thrombin was added. pH 7·4. Temperature 37° C.

From Table 1 it is seen that the addition of toluidine blue or $BaSO_4$ both of which bind heparin reduced the thrombin clotting time of heparinized plasma but were without effect on "stored" plasma. From these results it appears that the prolonged clotting time of "stored" plasma is not due to heparin.

The delayed thrombin clotting time of stored plasma might be due to the loss of a plasma component which accelerates the thrombin fibrinogen reaction as postulated by Ratnoff (1954). This possibility was tested by adding defibrinated fresh plasma to stored plasma.

TABLE 2.

Influence of defibrinated normal plasma on the thrombin clotting time (secs.) of "stored" and fresh plasma.

Plasma Plus	"Stored" Plasma	Fresh Plasma
0·2 ml. 0·14 M-NaCl containing 0·01 M sodium oxalate	120	13
0·2 ml. defibrinated fresh plasma	81	13

Experimental conditions similar to those given for Table 1.

The results given in Table 2 indicate that defibrinated fresh plasma decreased the thrombin clotting time of stored plasma only to a very slight degree. No evidence for a plasma accelerator of the thrombin fibrinogen reaction was therefore found.

Since neither inhibiting factors nor lack of accelerating factors explain the prolonged thrombin clotting time of stored plasma, a change of the fibrinogen molecule had to be considered. During storage increase of pH to values of 8-9 occurs. To see whether any chemical changes in the fibrinogen molecule took place, determinations of amide N and hexosamine were carried out in the belief that these radicals could be removed by alkaline hydrolysis. The hexosamine content of fibrin isolated from fresh plasma and from "stored" plasma were identical; being 0·38 p.c., calculated as glucosamine. The amide N contents were 1·41 p.c. and 1·37 p.c. respectively. From these figures it is apparent that the concentration of these radicals was not influenced by storage. The concen-

TABLE 3.

Influence of 0·03 M pyrocatechol on the thrombin clotting time (secs.) of fresh and "stored" plasma and isolated fibrinogen.

Experiment	Thrombin Clotting Time of:			
	Fresh Plasma		"Stored" Plasma	
	I	II	III	IV
1	13·5	8	68	14·5
2	15(240)	9	80(190)	17·5
3	18(210)	11	178(230)	21
4	10	8·5	26(200)	12
5	9·5(140)	5	22(130)	8

Reaction mixtures consisted of: experiments 1, 2, 3 : 0·4 ml. oxalated plasma (pH adjusted with N/10 acid); experiment 4 : fibrinogens isolated by ether precipitation: experiment 5: fibrinogens isolated by ammonium sulphate precipitation. 0·1 ml. veronal buffer pH 7·3. 0·1 ml. thrombin, pH 7·3, Temp. 28° C. The mixtures under columns II and IV contained 0·03 M pyrocatechol. Figures in brackets denote mg. p.c. fibrinogen.

tration of fibrinogen is not diminished during storage, since the same yield of fibrin as in fresh plasma is obtained. It seems likely that the change in the fibrinogen molecule is of a physical nature. It was shown previously that phenols affect the surface structure of fibrinogen (Fantl and Ebbels, 1953). It was therefore of interest to test the influence of pyrocatechol on the reactivity of the fibrinogen of "stored" plasma.

It can be seen from Table 3 that the thrombin clotting times of both fresh and "stored" plasma as well as fibrinogen isolated from these sources are shortened by 0·03M pyrocatechol. At a pH of 7·3 the shortening of the thrombin clotting time of the "stored" plasma is considerably greater than that of fresh plasma. However, in no case did pyrocatechol reduce the thrombin clotting time to the level of that of fresh plasma. In further experiments thrombin was incubated with "stored" plasma and after varying incubation times pyrocatechol was added. Such results are shown in Table 4.

TABLE 4.

Thrombin clotting times (secs.) of "stored" plasma incubated with thrombin for varying periods before the addition of pyrocatechol.

Incubation Time (seconds)	Thrombin Clotting Time	Thrombin Clotting Time After Incubation (0·03 M pyrocatechol-added)
0	205	25
60	—	7
120	—	9

Reaction mixtures similar in composition to those given in Table 3.

The results shown in Table 4 indicate that the initial reaction between thrombin and fibrinogen of "stored" plasma takes place, but the appearance of a clot is delayed. Pyrocatechol overcomes this lag. In order to obtain more information on the nature of the fibrinogen alteration during storage of plasma, the relationship between pH and thrombin clotting time was determined. The results are given in figures 2 and 3.

From figures 2 and 3 it is seen that pyrocatechol affected the thrombin clotting time to a lesser degree at pH below 7 than above that pH. This effect is particularly marked in the case of "stored" plasma. These results suggest that the prolonged thrombin clotting time of fibrinogen in stored plasma is due to hydration of the molecule. Pyrocatechol and H ions have a similar effect on dehydration which precedes fibrin separation.

It would appear from the presented experiments that due to the alkaline pH of the stored plasma the surface structure of the fibrinogen molecule is altered. Such fibrinogen still reacts with thrombin but the subsequent steps leading to fibrin separation are inhibited.

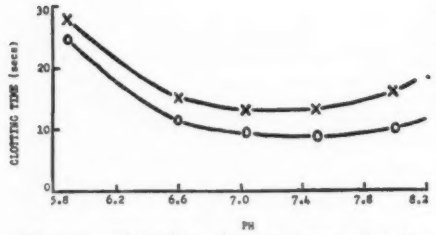

Fig. 2. Thrombin clotting times of fresh human oxalated plasma at various pH. Reaction mixtures consisted of: (1) 0·4 ml. plasma, 0·1 ml. veronal buffer and 0·1 ml. thrombin in 0·15M NaCl, temp. 28° C. X — (2) the same as (1) except that veronal buffers contained 2 p.c. pyrocatechol ○ —.

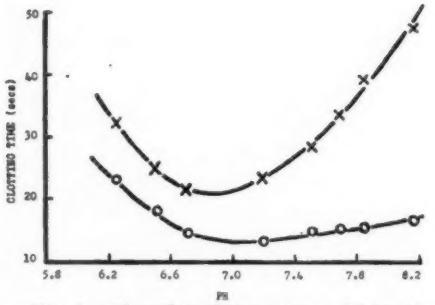

Fig. 3. Thrombin clotting times of "stored" human oxalated plasma at various pH. Experimental conditions and signs similar to those given in Fig. 2.

In order to overcome the damaging influence of OH ions on fibrinogen, normal plasmas were stored under a variety of conditions. The results are presented in Table 5.

TABLE 5.

Influence of storage conditions on fibrinogen reactivity towards thrombin.

Storage Conditions	Thrombin in Units	
	Added	Observed
A. *Oxalated plasma containing merthiolate*		
1. Air pH 9·0	1·25	0·1
2. Air pH 8·8	1·18	0·1
3. Hydrogen pH 8·7	1·33	0·40
4. Air pH 8·3	1·25	0·62
5. Nitrogen pH 8·0	1·33	0·41
6. Vacuum 4 mm. Hg. pH 7·6	1·40	0·63
7. Carbon dioxide pH 6·1	1·18	1·18
8. Carbon dioxide pH 6·1	1·25	1·25
B. *Citrated plasma containing merthiolate*		
1. Air pH 9·1	1·50	0·40
2. Air pH 8·8	1·05	0·35
3. Air pH 8·4	1·60	0·30
4. Air, HCl pH 7·0	1·60	1·05
5. Air, HCl pH 6·4	1·60	1·25
6. Carbon dioxide pH 6·5	1·60	1·33
7. Carbon dioxide pH 6·1	1·50	1·10
8. Carbon dixoide pH 6·3	1·05	1·00

From Table 5 it is apparent that plasma stored at room temperature at pH of 6·1-6·5 shows a normal thrombin clotting time. Carbon dioxide atmosphere had the same effect as acidification in air. It follows that preservation is due to the H ions and not to the exclusion of oxygen.

Having found means to preserve fibrinogen reactivity during storage it was of interest to study the effect of storage at an acid pH on other unstable clotting factors.

Results of experiments concerning prothrombin accelerator (proaccelerin) are given in Tables 6 and 7.

TABLE 6.

Influence of storage condition on the prothrombin clotting time of human citrated plasma.

Storage Condition	Prothrombin Time in Seconds	
	(a) without Ba-plasma	(b) with Ba-plasma
Air pH 8·4	76	17·5
Air HCl pH 6·9	19	12
Air HCl pH 6·4	15·5	12
CO_2 pH 6·6	16	13
CO_2 pH 6·3	15	12·5
Fresh plasma	10-12	10-12

Reaction mixtures consisted of: 0·1 ml. 0·025 M calcium chloride, 0·1 ml. 6 p.c. human brain extract in 0·15 M sodium chloride plus:

(a) 0·1 ml. plasma.

(b) 0·05 ml. plasma, 0·05 ml. fresh normal $BaSO_4$ treated plasma. Techniques of Quick (1938) and Quick and Stefanini (1948).

The results in Table 6 indicate that whereas plasma stored at pH above 8 has lost prothrombin accelerator, this can be prevented at pH 6·3-6·9.

Quantitative determinations of prothrombin accelerator in plasma stored in carbon dioxide atmosphere were made by an assay procedure in which the effect of prothrombin accelerator on the conversion of isolated prothrombin was measured. The results obtained are shown in Table 7.

TABLE 7.

Influence of normal and carbon dioxide stored plasma on the conversion of isolated prothrombin.

	Incubation time for max. thrombin formation (min.)	Thrombin clotting time (seconds)
0·015 ml. prothrombin solution	>5	>60
ditto plus 0·0015 ml. normal plasma	4	15
ditto plus 0·0030 ml. normal plasma	3	13
ditto plus 0·0015 ml. CO_2 stored plasma	7	13
ditto plus 0·0030 ml. CO_2 stored plasma	4	13

Testing was carried out according to Fantl (1954a).

As can be seen from Table 7 the isolated prothrombin preparation could not be converted to thrombin. The addition of 20 p.c. of plasma stored at pH 6·3 had the same accelerating effect as 10 p.c. fresh plasma. Preservation was the same at an acid pH whether the atmosphere was air or carbon dioxide.

Finally, the activity of alpha-prothromboplastin (anti-haemophilic factor) in "stored" plasma was measured by the rate of formation and yield of thrombin.

TABLE 8.

Rate of formation and yield of thrombin in "stored" citrated plasma.

Conditions of storage	Maximum Thrombin Yield	
	Incubation time in minutes	Yield of thrombin in units/ml. plasma
Air pH 8·4	>30	0
Air HCl pH 6·9	14·5	450
CO_2 pH 6·6	14·5	320
CO_2 pH 6·3	15	340
Fresh Plasma	7-11	414-450

Reaction mixtures according to method of Fantl, 1954b.

The results in Table 8 indicate that whereas no thrombin formation occurred in citrated plasma stored at a pH of 8·4, considerable activity remained at pH 6·3-6·9. This test, however, will only give reliable results for alpha-prothromboplastin if all the other clotting components are present in optimal concentration. Therefore the activity of alpha-prothromboplastin was estimated by the corrective effect of plasma stored in a carbon dioxide atmosphere on a fresh plasma completely deficient in alpha-prothromboplastin taken from an alpha-haemophilic patient.

TABLE 9.

Comparison of the effect of fresh human plasma and CO_2 stored plasma on the rate of thrombin formation in alpha-prothromboplastin deficient plasma.

p.c. of added Plasma	Addition of	
	Fresh citrated plasma	Citrated CO_2 stored plasma
*	Incubation time for max. thrombin yield (minutes)	Incubation time for max. thrombin yield (minutes)
50	16	—
25	18	18
10	22	25

*Normal plasma gave a maximum thrombin yield in 14 minutes. The final clotting time was 20 seconds.

From Table 9 it is seen that the addition of plasma stored in CO_2 atmosphere to a plasma completely deficient in alpha-prothromboplastin had almost the same effect as fresh normal plasma. In contrast plasma which had been stored at room temperature in air for 14 days was free of alpha-prothromboplastin. Thromboplastin generation tests of plasma stored at an acid pH are given in Table 10.

TABLE 10.

Comparison of thromboplastin generation in fresh and "stored" plasma.

Volume of Al-plasma in mixture	Fresh Citrated Al-plasma		CO_2 Stored Citrated Al-plasma	
	Incubation Time (mins.)	Clotting Time (secs.)	Incubation Time (mins.)	Clotting Time (secs.)
0·12 ml.	3	9	3	9
0·02 ml.	4	10	4	10
0·006 ml.	8	15	8	23
0·003 ml.	12	21	—	—

Reaction mixtures consisted of: 0·06 ml. normal human serum (free of prothrombin). Al-plasma as given in table. Test of Biggs and Douglas (1953) as modified by Bell and Alton (1954).

From Table 10 it is seen that the plasma stored in CO_2 atmosphere showed only slightly less alpha-prothromboplastin activity than fresh normal plasma. The results indicate that it is possible to preserve alpha-prothromboplastin in plasma stored for four weeks at room temperature provided that the pH is kept at 6·1-6·5.

DISCUSSION.

It has been shown in the present experiments that the hydrogen ion concentration of the plasma is the major factor in the preservation of certain clotting factors during storage. This explains why workers who used closed vessels for storage found greater stability of the clotting factors than those who stored in open vessels, and it explains also why acid citrate used as anticoagulant gives better preservation of the plasma components than sodium oxalate. The stability of the clotting factors is usually greater in animal plasma than in that of man, as has been noted for alpha-prothromboplastin by Spaet and Garner (1955) and for prothrombin accelerator by Murphy and Seegers (1948).

Regarding the individual clotting factors the present experiments have confirmed the observation that all the fibrinogen of oxalated or citrated plasma stored for one month at 18-20° C. in open vessels can be recovered as fibrin. However, the thrombin clotting time measured at pH 7·4 was greatly delayed if the OH ion concentration had increased to above pH 8 during storage. The delay was not due to increased antithrombic activity nor to the appearance of

heparin, because the addition of toluidine blue or barium sulphate, both of which bind heparin, did not alter the thrombin clotting time of stored plasma. Further, the addition of fresh defibrinated plasma did not correct the clotting time of stored plasma. This does not support the assumption that a plasma factor which accelerates the thrombin fibrinogen reaction had been lost during storage. No chemical difference could be detected between fibrinogen or fibrin isolated from stored plasma and that from fresh plasma. It could be shown that thrombin-fibrinogen interaction occurred in stored plasma, but the step leading to fibrin separation was inhibited. Pyrocatechol greatly accelerated this step. The experiments suggest that a change takes place in the surface configuration of the fibrinogen molecule during storage at alkaline pH. This is responsible for the delayed thrombin clotting time. Support for the assumption that the effect is due to OH ions is given by the fact that storage of plasma at a pH of 6·1-6·5 whether under aerobic or anaerobic conditions fully preserved the reactivity of fibrinogen in oxalated or citrated plasma.

Prothrombin accelerator activity is also lost during storage under conditions of OH ion increase. At pH 6·5 about 50 p.c. of the activity was preserved.

There is considerable divergence of opinion about the loss of alpha-prothromboplastin activity in stored plasma. Thus Taylor *et al.* (1944) found all the potency remained after 6 months at room temperature, whereas Collins (1955) detected about 50 p.c. activity after one week's storage in the refrigerator. The results of several different tests show that approximately 80 p.c. of the original activity could be recovered after one month's storage in citrate at a pH of 6·1-6·3, whereas no activity was found under conditions of hydroxyl ion increase at room temperature after 14 days. From this it would appear that the loss in alpha-prothromboplastin is also due to hydroxyl ions. Oxygen plays no role in this process.

It is not practical to use an anticoagulant which will give an acidity of pH $6{\cdot}5 \pm 0{\cdot}4$ for the preservation of whole blood because this would lead to considerable haemolysis. It is therefore necessary to separate plasma from cellular elements first, and adjust the pH in the isolated plasma. This can be carried out with mineral acid. However, for practical purposes it is suggested that carbon dioxide be used, since this does not result in volume increase and the excess is lost on the opening of the vessel, thus providing a liquid plasma suitable for transfusion. There is a need for the preservation of clotting factors since large volumes of plasma are often required in bleeding tendencies, and the usual method is to keep the plasma in a deep freeze. With the suggested procedure this is unnecessary.

SUMMARY.

The prolonged thrombin clotting time of sterile human oxalated or citrated plasma stored in open vessels at room temperature is not due to an increase

of antithrombin, to heparin-like substances, nor to loss of thrombin-accelerating factors.

It is due to a physical change of the surface structure of fibrinogen resulting from storage at an alkaline pH. Normal fibrinogen reactivity can be preserved by storage at pH 6·1-6·5.

The losses of prothrombin accelerator (proaccelerin) and alpha-prothromboplastin (anti-haemophilic factor) during storage of human plasma are also due to hydroxyl ions. The former can be partly, and the latter almost completely prevented by storage at a pH of 6·1-6·5.

Either mineral acid or carbon dioxide can be used for the preservation of the plasma clotting factors because the process is independent of oxygen. For transfusion purposes storage of plasma in carbon dioxide is suggested.

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SALMONELLA BACTERIOPHAGES

4. SOME BACTERIOPHAGES OF S. *BOVIS-MORBIFICANS*[1]

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In the bacteriophage grouping scheme for S. *bovis-morbificans* proposed by Atkinson (1956a), five bacteriophage groups were suggested and each was represented by a different indicator strain. The lysogenicity patterns of the five indicator strains were investigated by Atkinson (1956b) who found that three strains each carried at least one phage and two strains each carried at least two phages. As a non-lysogenic strain of S. *bovis-morbificans* was not available for cultivation of these phages, tests on all possible pairs among the five indicator strains were carried out to determine the best pair for production of each phage (Atkinson, 1956b). Using the selected pairs, crude preparations of the seven phages were made and appeared to represent five different types of S. *bovis-morbificans* phage. All the crude preparations contained more than one type of phage and therefore required treatment to free them from unwanted phage, before proceeding further with their subdivision into types. The present paper deals with improved preparations of the seven phages, the determination of some of their characteristics and their allocation to types.

Materials and Methods.

Preparation of the phages.

The seven phages under investigation were obtained from the five lysogenic indicator strains of S. *bovis-morbificans* by Atkinson (1956b) who labelled them according to the pairs of strains used in their production. Phage BM17/18 came from strain 17 (group 2), phage BM45/18 from strain 45 (group 3), phage BM18/8 from strain 18 (group 4), phages BM14/18 and BM14/8 from strain 14 (group 5) and phages BM8/18 and BM8/14 from strain 8 (group 6). A crude preparation of each phage was made by growing the selected pair of strains together on agar and extracting the mixed growth as described by Atkinson (1956b). Extracts of the pairs 17/18, 45/18 and 14/18 contained mainly the required phage from strains 17, 45 and 14 respectively which were resistant to the phage of the sensitive strain 18. In the pairs 18/8, 14/8, 8/18 and 8/14 each strain was sensitive to the phage from the other strain. Extracts of these pairs therefore contained much phage other than

[1] This work was assisted by a grant from the National Health and Medical Research Council, Canberra.

that required. The extracts were freed from bacteria by heating or filtering through a gradocol membrane, and some were also treated with BM14/18 antiserum to inactivate some of the unwanted phage. Sufficient antiserum was added to give a final concentration of 1/20 and the mixture was heated at 48° C. for 1 hour. Each extract was then inoculated into a young broth culture of the corresponding sensitive strain which, after incubation and centrifuging, was diluted, and plated out with the corresponding sensitive strain to obtain isolated plaques. Single plaques were picked off into a young broth culture of the corresponding sensitive strain which, after incubation and centrifuging, was heated or filtered, treated if necessary with BM14/18 antiserum, and constituted the stock phage preparation used in further investigations.

Investigation of phages.

Lysis tests were done by the method of Craigie and Yen (1938). Citrate sensitivity was determined by the method of Adams (1952) and urea sensitivity by the method of Burnet (1933). Heat sensitivity tests and antiserum neutralisation tests were carried out as described by Atkinson and Bullas (1956) for the phages of *S. adelaide*, except that lysis tests had to be done with the sensitive strain corresponding to the phage under test. Plaque size and morphology were determined in lysis tests on the corresponding sensitive strain on the standard nutrient agar used throughout our work, consisting of Oxoid nutrient broth with 10 p.c. Oxoid yeast extract and 1 p.c. agar added.

RESULTS.

Preparation of the phages.

Phages BM14/18 and BM8/18 were the strongest and most easily prepared of the seven phages. Crude preparations produced isolated plaques at dilutions between 10^{-5} and 10^{-6}. Single plaques of each phage were picked off and cultivated on strain 18 in broth. The broths, after centrifuging, were heated at 70° C. for 30 minutes to free them from bacteria and also from the small amounts of the heat sensitive phage BM18/8 coming from strain 18. These heated broths constituted the stock phages BM14/18 and BM8/18 and contained no contaminating phage.

Phages BM17/18 and BM45/18 were weaker phages than BM14/18 and BM8/18 and isolated plaques appeared from crude preparations at dilutions of 10^{-2} to 10^{-3}. Isolated plaques of each phage were picked off into broth with strain 18. After incubation and centrifugation the broths were freed from bacteria by gradocol filtration and constituted the stock phages BM17/18 and BM45/18. These two stock phages were contaminated with a small amount of phage BM18/8 coming from the sensitive strain 18. At a dilution of 10^{-1} this phage was no longer active but the phages BM17/18 and BM45/18 produced confluent to semi-confluent lysis.

Phages BM18/8, BM14/8, and BM8/14 were the most difficult of the seven phages to prepare as they were always accompanied in crude preparations by considerable amounts of one, two or even three other phages. An analysis of the phage content of these preparations was made by testing them on the three indicator strains 18, 14 and 8. Strain 18 was sensitive to phages BM14/18,

BM8/18 and BM8/14, strain 14 was sensitive only to phage BM8/14, and strain 8 was sensitive to phages BM18/8 and BM14/8 (Atkinson, 1956). Analysis showed that crude preparations of phage BM18/8 contained also phages BM8/18 and BM8/14 from strain 8, crude preparations of phage BM14/8 contained also phage BM8/14 from strain 8 and crude preparations of phage BM8/14 contained also phage BM14/8 from strain 14. The last two preparations also contained the two phages BM14/18 and BM8/18 given off by strains 14 and 8 during growth. Isolation of a single phage type from such mixtures was difficult. The crude preparations were heated at 56° C. for 30 minutes to destroy bacteria, treated with BM14/18 antiserum to neutralise the unwanted phages BM14/18 and BM8/18 and then enriched for the required phage by cultivation in broth with the corresponding sensitive strain. Analysis of these broths, after filtration and treatment with BM14/18 antiserum, showed that they all contained two types of phage of which the required phage was present in higher concentration and could be obtained as isolated plaques at dilutions from 10^{-1} to 10^{-2}. Single plaque preparations were made, filtered through a gradocol membrane and treated with BM14/18 antiserum to neutralise phage BM14/18 or BM8/18 from strain 14 or 8. These sterile preparations constituted the stock phages BM18/8, BM14/8 and BM8/14. They always contained small amounts of contaminating phage from the corresponding sensitive strains on which they were cultivated.

Investigation of the phages.

Lysis tests on the five indicator strains.—The results of lysis tests with the seven phages on the five indicator strains are given in Table 1.

TABLE 1.

Lysis tests with the seven S. bovis-morbificans *phages on the five indicator strains.*

Phage tested	Lysis on				
	Strain 17	Strain 45	Strain 18	Strain 14	Strain 8
BM17/18	−	−	+	+	+
BM45/18	+	−	+	+	−
BM18/8	tr	−	tr	tr	+
BM14/18	+	+	+	−	−
BM14/8	tr	−	tr	tr	+
BM8/18	+	+	+	−	−
BM8/14	+	−	+	+	tr

+ = confluent or semi-confluent lysis or many plaques.
tr = very few plaques.
− = no lysis.
Phage BM17/18 was used undiluted. The other phages were used at a dilution of 10^{-1}.

Different lysis patterns were given by the four phages BM17/18, BM45/18, BM18/8, and BM14/18. Phages BM18/8, BM14/8 and BM8/14 gave somewhat

similar lysis patterns in Table 1, but the weak reactions (tr) were due to the contaminating phage from the lysogenic propagating strain. The lysis pattern of each phage can be seen if the tr reactions are ignored. The lysis patterns of phages BM18/8 and BM14/8 then appear the same and phage BM8/14 gives a different pattern.

From the results in Table 1 four different lysis patterns emerged and were represented by phages BM17/18 with an individual pattern, BM45/18 and BM8/14 with similar patterns, BM14/18 and BM8/18 with similar patterns and BM18/8 and BM14/8 with similar patterns.

Plaque characteristics.—Plaques of phage BM17/18 on strain 18 were very small and clear with irregular edge and small spot of central growth, diameter 0·10-0·25 mm. Phage BM45/18 on strain 18 produced very small, hazy, irregular edged plaques covered with a film of bacterial growth, diameter 0·10-0·15 mm. Phages BM14/18 and BM8/18 produced on strain 18 somewhat similar plaques, larger than those produced by any of the other phages, diameter 0·15-0·5 mm. The plaques consisted of a large central spot of bacterial growth surrounded by a well defined clear ring with irregular edge. Phages BM18/8 and BM14/8 on strain 8 produced similar plaques which were very small and clear with irregular edge, diameter less than 0·05 mm. Phage BM8/14 produced very small plaques with a small spot of central growth surrounded by a clear ring with a regular edge, diameter less than 0·05 mm. Five plaque types were thus distinguished among the seven phages and were represented by phages BM17/18, BM45/18 and BM8/14 each with distinctive plaques, BM18/8 and BM14/8 with similar and distinctive plaques, BM14/18 and BM8/18 also with similar and distinctive plaques.

Heat resistance.—Suitable dilutions of the seven phages were held at various temperatures between 55° C. and 90° C. Typical results are given in Table 2.

TABLE 2.

Heat resistance tests with the seven S. bovis-morbificans *phages.*

Phage tested	Unheated	Heated for 30 minutes at							
		55° C	60° C	65° C	70° C	75° C	80° C	85° C	90° C
BM17/18	+	+	−	−	−	0	0	0	0
BM45/18	+	+	±	−	−	0	0	0	0
BM18/8	++	+	+	−	−	0	0	0	0
BM14/18	++	++	++	++	++	+	±	±	−
BM14/8	+	+	+	−	−	0	0	0	0
BM8/18	++	++	++	++	++	+	+	±	−
BM8/14	+	+	+	±	−	0	0	0	0

++ = confluent lysis.
+ = semi-confluent lysis or many plaques.
± = few plaques.
− = no lysis.
0 = not done.

Phage BM17/18 was the most heat sensitive and was inactive after exposure to 60° C. Phages BM45/18, BM18/8 and BM14/8 were less heat sensitive and survived 60° C. but were inactivated at 65° C. Phage BM8/14 survived to some extent at 65° C. but was inactivated at 70° C. Phages BM14/18 and BM8/18 were resistant to heat and survived 85° C. but were inactivated at 90° C. On heat sensitivity therefore the phages formed four groups consisting of phage BM17/18 which was most sensitive, phages BM45/18, BM18/8 and BM14/8 which were moderately sensitive, phage BM8/14 which was moderately resistant and phages BM14/18 and BM8/18 which were heat resistant.

Citrate sensitivity.—Phage BM45/18 failed to produce lysis in the presence of 0·05M citrate whereas the other phages BM17/18, BM18/8, BM14/18, BM14/8, BM8/18 and BM8/14 were unaffected.

Urea sensitivity.—Treatment with urea greatly reduced the activity of phage BM45/18 and partly reduced the activity of phages BM18/8 and BM14/8. The other phages BM17/18, BM14/18, BM8/18 and BM8/14 were unaffected.

Antiserum neutralisation tests.—Antiserum was prepared in a rabbit against phage BM14/18. After two courses of immunisation each involving 20 ml. of full strength phage, the serum in dilutions up to 1/1280 prevented lysis by phage BM14/18. The serum was tested against the other six phages and typical results are given in Table 3.

TABLE 3.

Neutralisation tests with the seven S. bovis-morbificans *phages and BM*14/18 *antiserum.*

Phage tested	Strain used in test	BM14/18 antiserum concentration					
		1/20	1/80	1/320	1/1280	1/2560	None
BM17/18	strain 18	+	+	+	+	+	+
BM45/18	strain 18	+	+	+	+	+	+
BM18/8	strain 8	+	+	+	+	+	+
BM14/18	strain 18	−	−	−	−	tr	+
BM14/8	strain 8	+	+	+	+	+	+
	strain 14	tr	tr	tr	tr	tr	tr
BM8/18	strain 18	−	−	−	−	tr	+
BM8/14	strain 14	+	+	+	+	+	+
	strain 8	tr	tr	tr	tr	tr	tr

+ = numerous plaques.
tr = very few plaques.
− = no lysis.

Phage BM8/18 was neutralised to much the same extent as phage BM14/18. These two phages were therefore very closely related serologically. The other five phages were unaffected by the BM14/18 antiserum and were therefore not related serologically to phage BM14/18. For phages BM14/8 and BM8/14 tests were made on both strains 8 and 14 to show the action of the serum on the

TABLE 4.

Lysis tests with the seven S. bovis-morbificans *phages and members of the* Enterobacteriaceae.

Test organism	No. of strains	Phage used in test						
		BM17/18	BM45/18	BM18/8	BM14/18	BM14/8	BM8/18	BM8/14
S. paratyphi A	1	−	−	−	−	−	−	−
S. paratyphi B	1	−	−	−	−	−	−	−
	1	−	+	−	−	−	−	−
S. typhi-murium	2	+	−	−	−	−	−	−
S. derby	2	−	−	−	−	−	−	−
S. muenchen	2	−	−	−	±	−	±	−
S. potsdam	1	−	−	−	−	−	−	−
S. blegdam	2	−	−	−	−	−	−	−
S. enteritidis	1	±	±	−	−	−	−	−
	1	−	−	−	−	−	−	−
S. anatum	2	−	−	−	−	−	−	−
S. worthington	2	−	−	−	−	−	−	−
S. aberdeen	1	−	−	−	−	−	−	+
S. carrau	1	−	−	−	−	−	−	−
S. onderstepoort	1	−	−	−	−	−	−	−
S. newington	2	−	−	−	−	−	−	−
S. senftenberg	1	−	−	−	−	−	−	−
S. adelaide	6	−	−	−	−	−	−	−
S. alachua	1	−	−	−	−	−	−	−
S. monschaui	1	−	−	−	−	−	−	−
S. waycross	4	−	−	−	−	−	−	−
Ballerup (*S. ballerup*)	1	−	−	−	−	−	−	−
Bethesda Nall	1	−	−	−	−	−	−	−
Bethesda Mdl	1	−	−	−	−	−	−	−
Arizona 699-52	1	−	−	−	−	−	−	−
E. coli 0 111	3	−	−	−	−	−	−	−
E. coli 0 55	1	−	−	−	−	−	−	−
E. freundii	1	−	−	−	−	−	−	−
Sh. boydii type 1	1	−	−	−	−	−	−	−
Sh. flexneri type 1A	1	−	−	−	−	−	−	−
Sh. dispar I	1	−	−	−	−	−	−	−
Pr. morgani	1	−	−	−	−	−	−	−
S. bovis-morbificans (strain used in preparing phage)		+	++	++	++	++	++	++

++ = confluent to semi-confluent lysis.
+ = many plaques.
± = few plaques.
− = no lysis.
Phages BM17/18 and BM18/8 were used undiluted.
Phages BM45/18, BM14/18, BM14/8, BM8/18 and BM8/14 were used at dilution of 10^{-1}.

two phages in these preparations, the contaminating phage from the lysogenic propagating strain giving only weak (tr) reactions.

Lysis tests on members of the Enterobacteriaceae.—Lysis tests with the seven phages were done on a selection of strains from the Salmonella group and other groups of the *Enterobacteriaceae* previously used to test the range of action of the phages of S. *adelaide* and S. *waycross* (Atkinson and Geytenbeek, 1953;

Atkinson and Carter, 1953; Atkinson and Bullas, 1956). No lysis of any of these strains occurred with phages BM18/8 and BM14/8. *S. typhi-murium* and *S. enteritidis* were lysed by phage BM17/18, *S. paratyphi* B and *S. enteritidis* by phage BM45/18, *S. aberdeen* by phage BM8/14 and *S. muenchen* by phages BM14/18 and BM8/18. Lysis was weak on all these strains. Thus the seven *S. bovis-morbificans* phages showed very little activity on this representative group of strains from the *Enterobacteriaceae* (see Table 4).

Allocation of the seven phages to types.

On heat sensitivity and neutralisation tests with BM14/18 antiserum two subdivisions appeared among the seven phages and consisted of phages BM14/18 and BM8/18 which were heat resistant and neutralised by BM14/18 antiserum, and phages BM17/18, BM45/18, BM18/8, BM14/8 and BM8/14 which were heat sensitive and not neutralised by BM14/18 antiserum. The heat resistant phages BM14/18 and BM8/18 closely resembled each other in all tests and were considered to represent a well-defined type of *S. bovis-morbificans* phage which was labelled type 5 as it was first found in *S. bovis-morbificans* strain 14 of group 5. Phage BM14/18 was selected as the prototype of type 5.

Among the five heat sensitive phages, BM17/18 was the most sensitive and BM8/14 the least sensitive. Phage BM17/18 gave a distinctive plaque and a characteristic lysis pattern which together with its heat sensitivity distinguished it from the other four phages. Phage BM17/18 was therefore considered to represent a recognisable type of *S. bovis-morbificans* phage which was called type 2 as it came from *S. bovis-morbificans* strain 17 of group 2. Phage BM17/18 was the prototype. Phage BM8/14 gave a lysis pattern similar to phage BM45/18 but in all other tests and in plaque morphology these phages differed from each other. Phage BM45/18 was more sensitive to heat and was also sensitive to citrate and urea neither of which had any effect upon phage BM8/14. Each of these phages was therefore considered to represent a recognisable type of *S. bovis-morbificans* phage. Phage BM45/18 was therefore allotted to type 3 as it came from *S. bovis-morbificans* strain 45 of group 3, and phage BM8/14, as it came from *S. bovis-morbificans* strain 8 of group 6, was allotted to type 6. These two phages were selected as the prototypes of types 3 and 6 respectively. The remaining two phages BM18/8 and BM14/8 appeared to be identical and differed from all the other phages in plaque morphology and lysis pattern. They were therefore considered to represent a recognisable type of *S. bovis-morbificans* phage first found in *S. bovis-morbificans* strain 18 of group 4 and therefore called type 4. Phage BM18/8 was taken as the prototype.

Five types of *S. bovis-morbificans* phage were thus distinguished among the seven phages and were labelled types 2, 3, 4, 5 and 6 according to the group number of the strain supplying the prototype phage.

Brief description of the five types of S. bovis-morbificans *phage.*

Type 2.—Prototype phage BM17/18. Plaques very small, clear with irregular edge and small central spot of bacterial growth; more sensitive to heat than types 3, 4, 5 and 6; unaffected by citrate or urea; unrelated serologically to type 5; lysed *S. bovis-morbificans* strains of groups 4, 5 and 6; produced by *S. bovis-morbificans* strains of group 2.

Type 3.—Prototype phage BM45/18. Plaques with irregular edge, very small and hazy, covered with a film of bacterial growth; moderately heat sensitive; sensitive to citrate and to urea; unrelated serologically to type 5; lysed *S. bovis-morbificans* strains of groups 2, 4 and 5; produced by *S. bovis-morbificans* strains of group 3.

Type 4.—Prototype phage BM18/8. Plaques minute (less than 0·05 mm. diameter) and clear with irregular edge; moderately heat sensitive; unaffected by citrate, but partially inactivated by urea; unrelated serologically to type 5; lysed *S. bovis-morbificans* strains of group 6 only; produced by *S. bovis-morbificans* strains of groups 4 and 5, accompanied in group 5 by type 5 phage.

Type 5.—Prototype phage BM14/18. Plaques larger than those of types 2, 3, 4 and 6, and consisting of a large central spot of bacterial growth surrounded by a clear ring with irregular edge; heat resistant; unaffected by citrate or urea; unrelated serologically to types 2, 3, 4 and 6; lysed *S. bovis-morbificans* strains of groups 2, 3 and 4; produced by *S. bovis-morbificans* strains of groups 5 and 6, accompanied in group 5 by type 4 phage and in group 6 by type 6 phage.

Type 6.—Prototype phage BM8/14. Plaques minute (less than 0·05 mm. diameter) with small central spot of bacterial growth surrounded by a clear ring with well defined edge; less sensitive to heat than types 2, 3 and 4; unaffected by citrate or urea; unrelated serologically to type 5; lysed *S. bovis-morbificans* strains of groups 2, 4 and 5; produced by *S. bovis-morbificans* strains of group 6, accompanied by type 5 phage.

DISCUSSION.

Of the seven *S. bovis-morbificans* phages, only the two heat resistant type 5 phages, BM14/18 and BM8/18, were obtained completely free from other contaminating phage by heating at 70° C. The other five phages were always contaminated to some extent by phage from the lysogenic sensitive strain upon which they had to be cultivated. Contaminating type 5 phage was inactivated with BM14/18 antiserum but other types of contaminating phage had to be removed as far as possible by dilution. Preparations adequate for the investigation of a number of characteristics, were obtained for the seven phages which, after investigation, were allocated to five types. The heat resistant type 5 phages resembled the type A phages of Burnet (1932) and Boyd (1950). They showed no serological relationship to the heat sensitive phages of types 2, 3, 4 and 6

which resembled the type B phages of Burnet and Boyd. The serology of these heat sensitive types is under investigation but results have been delayed by the difficulty in obtaining satisfactory phage preparations for antiserum production.

Carriage of type 5 heat stable phage together with heat labile type 4 or type 6 phage was observed in strains of groups 5 and 6. No obvious interference occurred between the two carried phages which both appeared to multiply at the same time if a sensitive strain was present.

The five types of *S. bovis-morbificans* phage showed very little activity on the other members of the *Enterobacteriaceae* tested here. They showed differential activity upon strains of *S. bovis-morbificans* and gave various lysis patterns on the five indicator strains of the bacteriophage grouping scheme proposed by Atkinson (1956a) who included a phage of each type in the grouping scheme to help in the definition of the phage groups. The phages actually used in the grouping scheme came from the preparations described in this paper.

SUMMARY.

Seven phages were obtained from the five lysogenic indicator strains of *S. bovis-morbificans* and tested for heat, citrate and urea sensitivity, lysis pattern, plaque morphology and neutralisation with BM14/18 antiserum. On the results of these tests the seven phages were allocated to five types numbered 2, 3, 4, 5 and 6 according to the phage groups of the indicator strains from which the phage types came. Type 5 phage was heat resistant and serologically distinct from the other types which were heat sensitive. Of the seven phages, one was allocated to type 2, one to type 3, two to type 4, two to type 5 and one to type 6. Each type of phage was briefly described and prototype phages for each type were designated.

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SALMONELLA BACTERIOPHAGES

5. SOME BACTERIOPHAGES OF *S. POTSDAM*[1]

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In an investigation of the lysogenicity and lysis patterns of 15 strains of *S. potsdam*, Atkinson and Bullas (1956c) found 13 lysogenic strains of which eight carried heat resistant phage and were placed in five groups (2-6). Numerous heat sensitive phages were also detected and most of the lysogenic strains carried more than one phage. The heat resistant phages were isolated and some of their characteristics are given in this paper.

Materials and Methods.

The phages investigated here were the eight heat resistant *S. potsdam* phages P4h/2, P5h/2, P8h/2, P9h/2, P12h/2, P13h/2, P14h/2 and P16h/2 isolated by Atkinson and Bullas (1956c) from *S. potsdam* strains 4, 5, 8, 9, 12, 13, 14 and 16 respectively. All the phages were cultivated upon the nonlysogenic sensitive *S. potsdam* strain 2. The stock phage preparations were prepared from isolated plaques and heated at 70° C. for 30 minutes.

Lysis tests were done by the method of Craigie and Yen (1938). Citrate sensitivity was determined by the method of Adams (1952) and urea sensitivity by the method of Burnet (1933). Antiserum neutralisation tests and heat sensitivity tests followed the methods used by Atkinson and Bullas (1956a) for investigation of the phages of *S. adelaide* except that *S. potsdam* strain 2 was used in all lysis tests. Plaque morphology was determined in lysis tests on the standard nutrient agar (Atkinson and Bullas, 1956a) on *S. potsdam* strain 2.

RESULTS.

Lysis patterns on S. potsdam *strains.*—Lysis tests with the eight phages were carried out on the 15 strains of *S. potsdam* described by Atkinson and Bullas (1956c). The results are given in Table 1 in which are grouped together the strains shown to carry heat resistant phage and the strains not shown to carry heat resistant phage.

The eight phages failed to lyse any of the eight strains 4, 5, 8, 9, 12, 13, 14 and 16 carrying heat resistant phage. Of the seven strains not shown to

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TABLE 1.

Lysis tests with eight S. potsdam *phages on* 15 *strains of* S. potsdam.

Phage tested	Lysis on														
	Strains carrying heat resistant phage.								Strains not carrying heat resistant phage						
	4	5	8	9	12	13	14	16	2	3	6	10	11	15	17
P4h/2	−	−	−	−	−	−	−	−	++	−	++	+	−	++	−
P5h/2	−	−	−	−	−	−	−	−	++	++	++	±	−	++	−
P8h/2	−	−	−	−	−	−	−	−	++	++	++	±	−	+	−
P9h/2	−	−	−	−	−	−	−	−	++	++	++	−	−	++	−
P12h/2	−	−	−	−	−	−	−	−	++	++	++	−	−	++	−
P13h/2	−	−	−	−	−	−	−	−	++	++	++	±	−	++	−
P14h/2	−	−	−	−	−	−	−	−	++	++	++	±	−	++	−
P16h/2	−	−	−	−	−	−	−	−	++	−	++	+	−	++	−

All phages diluted 10^{-1}.
++ = confluent lysis.
+ = many plaques.
± = a few plaques.
− = no lysis.

carry heat resistant phage, the three strains 2, 6 and 15, were lysed by the eight phages, strain 3 was lysed by all except phages P4h/2 and P16h/2, strain 10 was weakly lysed by all phages except P9h/2 and P12h/2, and strains 11 and 17 were not lysed by any phage. The eight phages were therefore restricted in range of activity on strains of *S. potsdam* and failed to lyse not only those strains carrying heat resistant phage but also certain other strains such as 11 and 17 not shown to carry heat resistant phage. Lysis by any of these phages indicated that the lysed strain was not carrying heat resistant phage. Failure of a strain to lyse with any of these phages was frequently, but not necessarily, due to carriage of heat resistant phage.

In lysis tests with undiluted phage, some of the eight phages showed an atypical clearing on certain strains not lysed by phage diluted 10^{-1}. This effect, which is under further investigation, was considered to be due to a lysin similar to those described by Boyd (1950) for certain phages of *S. typhi-murium*.

Citrate sensitivity.—Phages P9h/2 and P12h/2 were inactivated in the presence of 0·05M citrate which had no effect upon phages P4h/2, P5h/2, P8h/2, P13h/2, P14h/2 and P16h/2.

Urea sensitivity.—The activity of the eight phages was unaffected by treatment with urea.

Heat sensitivity.—Suitable dilutions of the eight phages were heated for 30 minutes at 70° C., 75° C., 80° C., 85° C. and 90° C. Typical results are given in Table 2.

TABLE 2.

Heat-resistance tests with eight S. potsdam *phages.*

Phage tested	Unheated	Heated at				
		70° C	75° C	80° C	85° C	90° C
P4h/2	++	++	++	++	+	−
P5h/2	++	++	++	++	+	−
P8h/2	++	++	++	++	±	−
P9h/2	++	++	++	++	+	−
P12h/2	++	++	++	+	±	−
P13h/2	++	++	++	++	+	−
P14h/2	++	++	++	++	±	−
P16h/2	++	++	++	+	+	−

++ = confluent lysis.
+ = semi-confluent lysis to many plaques.
± = few plaques.
− = no lysis.

All the phages resisted 80° C. but were partly inactivated at 85° C. and completely inactivated at 90° C. They were very resistant to heat.

Resistance to cold storage.—The eight phages showed no loss of activity after storage for ten months in a refrigerator at 2-4° C. After storage frozen at − 22° C. for eight months some loss of activity occurred with all phages except P5h/2 and P8h/2.

Plaque morphology.—The eight phages produced plaques varying in diameter from 0·3 to 1 mm. Large and small plaques were always observed together.

TABLE 3.

Neutralisation tests with eight S. potsdam *phages and two antisera.*

Phage tested	Antiserum and concentration tested							
	P4h/2				P8h/2			
	1/80	1/640	1/2560	None	1/20	1/80	1/320	None
P4h/2	−	−	−	++	0	0	0	0
P5h/2	−	−	−	++	0	0	0	0
P8h/2	−	−	−	++	−	−	±	+
P9h/2	−	−	−	+	−	−	+	++
P12h/2	−	−	−	+	0	0	0	0
P13h/2	−	−	−	++	−	−	±	++
P14h/2	−	−	−	+	−	−	±	++
P16h/2	−	−	−	+	−	−	±	+

++ = confluent to semi-confluent.
+ = numerous plaques.
± = a few plaques.
− = no lysis.
0 = not tested.

In general, the larger plaques consisted of a central area of bacterial growth surrounded by a more or less clear ring of lysis with irregular edge. Phages P9h/2 and P12h/2 produced larger areas of central growth than the other phages. Phages P13h/2 and P14h/2 differed from the other phages in producing plaques surrounded by a halo.

Antiserum neutralisation tests.—Antisera were easily prepared in rabbits against phages P4h/2 and P8h/2. The phages were tested for neutralisation with these two antisera. Typical results are given in Table 3.

The P4h/2 antiserum was tested against the eight phages and neutralised them all to the same extent as phage P4h/2 used to produce the serum. The P8h/2 antiserum was tested against the four phages P9h/2, P13h/2, P14h/2 and P16h/2

TABLE 4.

Lysis tests with eight S. potsdam *phages and members of the* Enterobacteriacaea.

Test organism	No. of strains	Phage used in test							
		P4h/2	P5h/2	P8h/2	P9h/2	P12h/2	P13h/2	P14h/2	P16h/2
S. paratyphi A	1	–	–	–	–	–	–	–	–
S. paratyphi B	2	–	–	–	–	–	–	–	–
S. typhi-murium	2	–	–	–	–	–	–	–	–
S. derby	2	–	–	–	–	–	–	–	–
S. muenchen	2	–	–	–	–	–	–	–	–
S. bovis-morbificans	5	–	–	–	–	–	–	–	–
S. blegdam	2	–	–	–	–	–	–	–	–
S. enteritidis	2	–	–	–	–	–	–	–	–
S. anatum	2	–	–	–	–	–	–	–	–
S. worthington	2	–	–	–	–	–	–	–	–
S. aberdeen	1	–	–	–	–	–	–	–	–
S. carrau	1	–	–	–	–	–	–	–	–
S. onderstepoort	1	–	–	–	–	–	–	–	–
S. newington	2	–	–	–	–	–	–	–	–
S. senftenberg	1	–	–	–	–	–	–	–	–
S. adelaide	6	–	–	–	–	–	–	–	–
S. alachua	1	–	–	–	–	–	–	–	–
S. monschaui	1	–	–	–	–	–	–	–	–
S. waycross	4	–	–	–	–	–	–	–	–
Ballerup (*S. ballerup*)	1	–	–	–	–	–	–	–	–
Bethesda Nall	1	–	–	–	–	–	–	–	–
Bethesda Mdl	1	–	–	–	–	–	–	–	–
Arizona 699-52	1	–	–	–	–	–	–	–	–
E. coli 0 111	3	–	–	–	–	–	–	–	–
E. coli 0 55	1	–	–	–	–	–	–	–	–
E. freundii	1	–	–	–	–	–	–	–	–
Sh. boydii type 1	1	–	–	–	–	–	–	–	–
Sh. flexneri type 1A	1	–	–	–	–	–	–	–	–
Sh. dispar I	1	–	–	–	–	–	–	–	–
Pr. morgani	1	–	–	–	–	–	–	–	–
S. potsdam strain 2	1	++	++	++	++	++	++	++	++

All phages used at a dilution of 10^{-1}.
++ = confluent to semi-confluent lysis.
– = no lysis.

and neutralised them to the same extent as phage P8h/2 used to produce the serum. The eight phages were therefore closely related serologically.

Lysis tests on members of the Enterobacteriaceae.—Lysis tests were carried out on a selection of strains representing various Salmonella types and other groups of the *Enterobacteriaceae*. The same group of strains was used previously to test the range of activity of the phages of S. *adelaide*, S. *waycross* and S. *bovis-morbificans* (Atkinson and Geytenbeek, 1953; Atkinson and Carter, 1953; Atkinson and Bullas, 1956a and b). The results of these tests are given in Table 4.

The eight phages failed to lyse any of the other members of the *Enterobacteriaceae* included in Table 4.

Allocation of the phages to types.—The eight S. *potsdam* phages formed a single well-defined group and resembled each other in heat resistance, resistance to cold storage, urea sensitivity, serology, general plaque size and morphology and failure to lyse strains of S. *potsdam* carrying heat resistant phage. They were therefore considered to represent a recognisable type of S. *potsdam* phage which was labelled type 2. Some variations occurred in details of plaque morphology, citrate sensitivity, and lysis pattern on strains of S. *potsdam* not carrying heat resistant phages. However, the creation of subtypes to accommodate these variations seemed unwarranted as yet.

Brief description of S. potsdam *phage type* 2.—Prototype phage P5h/2. Plaques variable in diameter, from 0·3 to 1 mm., consisting of a central spot of bacterial growth surrounded by a ring of lysis with irregular edge; resistant to heating for 30 minutes at 80° C., reduced at 85° C., and inactivated at 90° C.; resistant to cold storage; not sensitive to urea; occurred in S. *potsdam* strains 4 (group 2), 5 and 8 (group 3), 9 and 12 (group 4), 13 and 14 (group 5), and 16 (group 6) frequently accompanied by heat sensitive phage; failed to lyse strains of S. *potsdam* carrying heat resistant phage; failed to lyse a wide range of strains from the *Enterobacteriaceae*.

DISCUSSION.

The eight S. *potsdam* phages formed a serologically related group of very heat resistant phages and were therefore designated as a recognisable type of S. *potsdam* phage called type 2. The S. *potsdam* type 2 phage resembled in plaque morphology and serology the A type phages described by Burnet (1932) for strains of S. *paratyphi* A, B, and C, S. *enteritidis* and S. *typhi-murium* and by Boyd (1950) for S. *typhi-murium*. In our earlier work on S. *bovis-morbificans* (Atkinson and Bullas, 1956b) two heat resistant phages were isolated, and heat resistant phages have also been obtained from strains of S. *blegdam*, S. *enteritidis* and S. *muenchen*. All these heat resistant phages are now being compared and some general observations on the relationships within this larger and more heterogeneous group will be published in a separate paper.

SUMMARY.

Eight heat resistant phages isolated from eight strains of *S. potsdam* were found to resemble each other closely in general plaque morphology, serology, insensitivity to urea and failure to lyse strains of *S. potsdam* carrying heat resistant phage. They thus formed a single, well-defined group which was called *S. potsdam* phage type 2. Minor variations occurred in citrate sensitivity, some details of plaque morphology and lysis pattern on strains of *S. potsdam* not carrying heat resistant phage. These variations were not considered adequate, as yet, for the creation of subtypes.

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LYSOGENICITY AND LYSIS PATTERNS IN THE SALMONELLAS

10. *S. POTSDAM.*[1]

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Previous papers in this series recorded the lysogenicity and lysis patterns found among strains of *S. adelaide*, *S. waycross* and *S. bovis-morbificans*. For each type, bacteriophage grouping schemes were proposed and phages carried by the groups were characterised (Atkinson *et al.*, 1952; Atkinson, 1955, 1956a; Atkinson and Geytenbeek, 1953; Atkinson and Carter, 1953; Atkinson and Bullas, 1956 a and b). Similar investigations were carried out on strains of *S. potsdam* and some of the results are reported here.

Materials and Methods.

Fifteen strains of *S. potsdam* were investigated. Fourteen of these strains were isolated in Australia and one was the type strain S37 (NCTC 3206). Of the 14 Australian strains, 13 (recorded by Atkinson, 1956b) came from the Salmonella Typing Centre of this laboratory and one was provided by Dr. M. M. Wilson of the Melbourne University, to whom our thanks are due. The sources of the 15 strains are given in Table 1.

Strains 2, 3, 6 and 11 came from unrelated human cases of gastro-enteritis, strains 5 and 8 from a sheep dying in a feeding test, strain 10 from a cow with gastro-enteritis, strain 12 from a chicken dying of pullorum disease, and strains 9, 13, 14, 15, 16 and 17 from lizards. The lizard strains were recorded by Lee and Mackerras (1955). Strain 4 came from the National Collection of Type Cultures, London, and was the only strain from outside Australia.

Lysogenicity was detected by the method described by Atkinson (1956c) for strains of *S. bovis-morbificans*. The 15 strains of *S. potsdam* were tested for lysogenicity in pairs in all possible combinations.

RESULTS.

The results of lysogenicity tests with all possible pairs of strains of *S. potsdam* are given in Table 2 in which the horizontal rows give the lysogenicity pattern of each strain on the other 14 strains and the vertical rows give the sensitivity of each strain to the lysogenic action of the other 14 strains.

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TABLE 1.

Sources of 15 *strains of* S. potsdam.

Strain no.	Year and place of isolation	Source
2	1944, N.S.W.	faeces of child with gastro-enteritis.
3	1950, N.S.W.	faeces of infant with gastro-enteritis.
4	Not from Australia	stock strain S37 (NCTC3206) from an acute case of human gastro-enteritis.
5	1950, Queensland	intestine of sheep dying during depleting of vitamin A.
6	1951, Queensland	faeces of infant with gastro-enteritis also involving *S. typhi-murium* and *Sh. sonnei*.
8	1950, Queensland	lymph node of sheep yielding strain 5 from intestine.
9	1951, Queensland	lizard dying after a few months in captivity.
10	1952, Queensland	colon contents of cow with marked gastro-enteritis.
11	1953, Victoria	faeces of human case of acute gastro-enteritis.
12	1952, South Australia	chicken dying of pullorum disease.
13	1954, Queensland	lizard with inapparent infection.
14	1954, Queensland	lizard with inapparent infection.
15	1954, Queensland	lizard with inapparent infection.
16	1954, Queensland	lizard with inapparent infection.
17	1954, Queensland	lizard with inapparent infection.

TABLE 2.

Lysogenicity tests on all possible pairs of strains of S. potsdam.

Test strain	Lysogenic activity on strain														
	2	3	4	5	6	8	9	10	11	12	13	14	15	16	17
2	0	−	−	−	−	−	−	−	−	−	−	−	−	−	−
3	+	0	+	±	+	±	+	+	+	+	+	+	+	+	−
4	+	−	0	−	+	−	−	−	−	−	−	−	+	−	−
5	+	+	−	0	+	−	−	−	−	−	±	+	+	−	−
6	−	−	−	−	0	−	−	−	−	−	−	−	−	−	−
8	+	+	−	−	+	0	−	−	−	−	+	+	+	−	−
9	+	+	+	+	+	+	0	−	+	−	+	+	+	−	−
10	+	+	+	+	+	+	+	0	+	+	+	+	+	+	−
11	+	+	−	+	+	+	−	−	0	−	+	+	+	±	−
12	+	+	+	+	+	+	−	−	+	0	+	+	+	−	−
13	+	+	+	+	+	+	+	−	+	+	0	−	+	+	±
14	+	+	+	+	+	+	+	−	+	+	−	0	+	+	±
15	+	+	+	+	+	+	+	−	+	+	+	+	0	+	+
16	+	+	±	+	+	+	+	±	+	+	+	+	+	0	+
17	+	±	−	±	+	±	−	−	±	−	+	+	+	+	0

+ = confluent or semi-confluent lysis to many plaques.
± = few plaques.
− = no lysis.
0 = not done.

Strains 2 and 6 showed no lysogenic activity on any strain. They were both lysed by the other 13 strains which were all therefore shown to be lysogenic. The lysogenicity patterns of the 13 lysogenic strains showed considerable variation. Strain 16 was lysogenic on all the other strains, and strains 3, 10 and 15

were each lysogenic on all except one of the other strains. In contrast, strain 4 was lysogenic on only one of the other lysogenic strains. Strains 5, 8, 9, 11, 12, 13, 14 and 17 showed various other patterns in which strain 5 resembled strain 8, strain 9 resembled strain 12 and strain 13 resembled strain 14. These results suggested that several different bacteriophage groups, associated with a number of different phages, occurred among the lysogenic strains.

Further to investigate the phages of the lysogenic strains, crude phage preparations were made by growing each strain with strain 2 on agar and extracting the mixed growth of each pair in broth as for the lysogenicity tests. Strain 2 was sensitive to phage from all the lysogenic strains and was itself nonlysogenic. The extract of each pair therefore contained phage from the lysogenic strain capable of multiplying on strain 2. These extracts, which were the crude phage preparations, were numbered after the pair of strains used in their production and further labelled P to indicate that they contained S. *potsdam* phages. P3/2 came from strains 3 and 2 grown together and contained phage from strain 3. The results of lysis tests with these crude phages on the 15 strains are given in Table 3.

TABLE 3.

Lysis tests with crude phage preparations on the fifteen strains of S. potsdam.

Crude phage	Lysis on strain														
	2	3	4	5	6	8	9	10	11	12	13	14	15	16	17
P3/2	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+
P4/2	+	+	−	−	+	−	−	+	−	−	−	−	+	−	−
P5/2	+	+	−	−	+	−	−	−	+	−	+	+	+	−	+
P8/2	+	+	−	−	+	−	−	−	+	−	+	+	+	−	+
P9/2	+	+	+	+	+	+	−	−	+	−	+	+	+	−	+
P10/2	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+
P11/2	+	+	+	+	+	+	−	−	−	−	+	+	+	+	+
P12/2	+	+	+	+	+	+	−	−	+	−	+	+	+	−	+
P13/2	+	+	+	+	+	+	+	−	+	+	−	−	+	+	+
P14/2	+	+	+	+	+	+	+	−	+	+	−	−	+	+	+
P15/2	+	+	+	+	+	+	+	−	+	+	+	+	−	+	+
P16/2	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+
P17/2	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−

+ = confluent lysis to plaques.
− = no lysis.

As expected, each crude phage preparation in Table 3 lysed all the strains affected by the corresponding lysogenic strain in Table 2. Unexpectedly, however, some of the crude phage preparations also lysed strains unaffected by their corresponding lysogenic strains in Table 2. Thus in Table 3, crude phages P3/2, P5/2, P8/2, P9/2, P10/2, P11/2 and P12/2 lysed strain 17 which was unaffected by strains 3, 5, 8, 9, 10, 11 and 12 in Table 2, and crude phage P17/2 lysed

strains 4, 9 and 12 unaffected by strain 17 in Table 2. Minor variations in lysis pattern occurred with other crude phages when compared with their corresponding lysogenic strains. These variations were probably due to differences in the concentration of phage in the crude phage preparations in Table 3 and in the extracts used in the lysogenicity tests in Table 2.

In Table 3 the crude phages gave many different lysis patterns. However, crude phages P5/2 and P8/2 gave identical patterns, P9/2 and P12/2 gave identical patterns and P13/2 and P14/2 gave identical patterns. These results supported the relationship between strains 5 and 8, strains 9 and 12, and strains 13 and 14 suggested by the lysogenicity patterns in Table 2.

To detect heat stable phages, the crude phage preparations were heated at 70° C. for 30 minutes and then tested on the 15 strains. The results of these tests are given in Table 4.

TABLE 4.

Lysis tests with crude phage preparations after heating at 70° C.

Crude phage	Lysis on strain														
	2	3	4	5	6	8	9	10	11	12	13	14	15	16	17
P3/2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
P4/2	+	−	−	−	+	−	−	−	−	−	−	−	+	−	−
P5/2	+	+	−	−	+	−	−	−	−	−	−	−	+	−	−
P8/2	+	+	−	−	+	−	−	−	−	−	−	−	+	−	−
P9/2	+	+	(+)	−	+	−	−	−	(+)	−	(+)	(+)	+	−	−
P10/2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
P11/2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
P12/2	+	+	(+)	−	+	−	−	−	(+)	−	(+)	(+)	+	−	−
P13/2	+	+	−	−	+	−	−	−	−	−	−	−	+	−	−
P14/2	+	+	−	−	+	−	−	−	−	−	−	−	+	−	−
P15/2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
P16/2	+	−	−	−	+	−	−	−	−	−	−	−	+	−	−
P17/2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

(+) = these reactions disappeared after heating at 75° C for 30 minutes.
\+ = confluent lysis to plaques.
− = no lysis.

The results in Table 4 showed the presence of heat resistant phage in the crude phage preparations P4/2, P5/2, P8/2, P9/2, P12/2, P13/2, P14/2 and P16/2 and indicated that strains 4, 5, 8, 9, 12, 13, 14 and 16 carried heat resistant phage which was not detected in strains 3, 10, 11, 15 and 17. The heated crude phages showed three different lysis patterns in which P5/2, P8/2, P13/2 and P14/2 lysed strains 2, 3, 6 and 15, P4/2 and P16/2 lysed strains 2, 6 and 15, and P9/2 and P12/2 lysed strains 2, 3, 4, 6, 11, 13, 14 and 15. Thus strains 5, 8, 9, 12 and 16 carrying heat resistant phage were resistant to lysis by all the heated phage preparations, but strains 4, 13 and 14 also carrying heat resistant phages were lysed by the heated phage preparations P9/2 and P12/2.

Strains 10 and 17 not shown to carry heat resistant phage were not lysed by any of the heated phage preparations. The results in Table 4 therefore suggested that no relation existed between carriage of heat resistant phage and sensitivity to lysis by the heated crude phages.

The heat resistant phages were isolated from single plaques which were cultivated on strain 2 and heated at 70° C. for 30 minutes. Each preparation was designated by the letter "h" accompanying the number of the crude phage from which it came. These preparations were considered to contain only one type of phage and were tested for lysis on the 15 strains. The results of these tests are given in Table 5.

TABLE 5.

Lysis tests with heat resistant phages.

Test phage	Lysis with strain														
	2	3	4	5	6	8	9	10	11	12	13	14	15	16	17
P4h/2	+	−	−	−	+	−	−	+	−	−	−	−	+	−	−
P5h/2	+	+	−	−	+	−	−	±	−	−	−	−	+	−	−
P8h/2	+	+	−	−	+	−	−	±	−	−	−	−	+	−	−
P9h/2	+	+	−	−	+	−	−	−	−	−	−	−	+	−	−
P12h/2	+	+	−	−	+	−	−	−	−	−	−	−	+	−	−
P13h/2	+	+	−	−	+	−	−	±	+	−	−	−	+	−	−
P14h/2	+	+	−	−	+	−	−	±	+	−	−	−	+	−	−
P16h/2	+	−	−	−	+	−	−	+	−	−	−	−	+	−	−

All phages used at a dilution of 10^{-1}.
+ = confluent or semi-confluent lysis.
± = a few plaques.
− = no lysis.

Except for P9h/2 and P12h/2, the purified phages gave lysis patterns closely resembling those of the heated crude phages from which they came. Similar lysis patterns appeared with phages P5h/2, P8h/2, P9h/2 P12h/2, P13h/2 and P14h/2 which lysed strains 2, 3, 6, 10 and 15. Phages P4h/2 and P16h/2 lysed strains 2, 6, 10 and 15 and failed to lyse strain 3.

The purified phages P9h/2 and P12h/2 (Table 5) gave different lysis patterns from the heated crude phages P9/2 and P12/2 (Table 4) as they failed to lyse strains 4, 11, 13 and 14. This difference was shown to be due to the presence of two different phages in the heated crude preparations P9/2 and P12/2. When these preparations were heated at 75° C. for 30 minutes they no longer lysed strains 4, 11, 13 and 14 but continued to lyse strains 2, 3, 6 and 15 and thus gave the lysis pattern found with the purified phages P9h/2 and P12h/2. The crude preparations therefore contained these heat resistant phages and also other phages which were moderately heat resistant and survived at 70° C. but not at 75° C. To avoid similar confusion in the future any prepara-

tion to be tested for presence of heat resistant phage will be held at 75° C. for 30 minutes.

In Table 5 all the strains shown to carry heat resistant phage failed to lyse with the eight phages. Carriage of heat resistant phage was thus directly correlated with resistance to lysis by heat resistant phages. The converse was not shown, however, as strains 11 and 17, not carrying heat resistant phages, were also resistant to the eight heat resistant phages.

Comparison of the lysis patterns of the crude phage preparations P5/2, P8/2, P9/2, P12/2, P13/2, P14/2 and P16/2 (Table 3) with the lysis patterns of the heat resistant phages P5h/2, P8h/2, P9h/2, P12h/2, P13h/2, P14h/2 and P16h/2 (Table 5) derived from them, showed that the crude preparations contained other phages besides these heat resistant phages which accounted for only part of their lysis patterns. For example the crude phage preparation P16/2 (Table 3) lysed strains 3, 4, 5, 8, 9, 11, 12, 13, 14 and 17 not lysed by the purified phage P16h/2 (Table 5). Strains 5, 8, 9, 12, 13, 14 and 16 therefore carried other heat sensitive phages lysing strains unaffected by the heat resistant phages. The crude phage preparation P4/2, however, gave a lysis pattern similar to that of the purified phage P4h/2 except for lysis of strain 3. Strain 4 has not yet been shown to carry more than one phage.

On the results so far given the 15 strains of *S. potsdam* were divisible into three sections consisting of strains 2 and 6 not shown to carry any phage, strains 3, 10, 11, 15 and 17 carrying only heat sensitive phage, strains 4, 5, 8, 9, 12, 13, 14 and 16 carrying heat resistant phage and, with the possible exception of strain 4, also carrying heat sensitive phage. Strains 2 and 6 constituted a well-defined group which was called group 1 and was non-lysogenic on the other 13 strains of *S. potsdam*, but was sensitive to lysis by these strains. Strains 3, 10, 11, 15 and 17 were heterogeneous in their lysogenicity patterns and no grouping seemed obvious. Each strain appeared to represent a different group. The phages of these strains are being isolated and studied and definite groups will be suggested when more information is available. Among the eight strains carrying heat resistant phage, strains 5 and 8 appeared the same, strains 9 and 12 appeared the same and strains 13 and 14 appeared the same. All these strains were different from strain 4 and also from strain 16. These strains were therefore further divided into five groups. Strain 4 was placed in group 2, strains 5 and 8 in group 3, strains 9 and 12 in group 4, strains 13 and 14 in group 5 and strain 16 in group 6. These groups all failed to lyse with phage P5h/2 representing the heat resistant phages and were distinguished by their lysogenicity patterns on the five indicator strains of these groups and on strain 2 (group 1). The indicator strains chosen were group 1 strain 2, group 2 strain 4, group 3 strain 5, group 4 strain 9, group 5 strain 13, and group 6 strain 16. Except on strain 2, the typical patterns of these groups were due only to heat sensitive phage. In Table 6 the lysogenicity pattern of the indicator strain of each group is given on the six indicator strains.

TABLE 6.

Lysogenicity patterns of indicator strains of the S. potsdam *bacteriophage groups* 1 *to* 6.

Strain tested	Lysis with phage P5h/2	Lysogenic activity on strain					
		2	4	5	9	13	16
		(group 1)	(group 2)	(group 3)	(group 4)	(group 5)	(group 6)
2	+	−	−	−	−	−	−
4	−	+	−	−	−	−	−
5	−	+	−	−	−	±	−
9	−	+	+	+	−	+	−
13	−	+	+	+	+	−	+
16	−	+	±	+	+	+	−

+ = confluent lysis to many plaques.
± = few plaques.
− = no lysis.

The lysogenicity pattern (horizontal row in Table 6) of each indicator strain was distinctive and allowed its recognition. The lysis patterns (vertical rows in Table 6) of the indicator strains 2, 9, 13 and 16 due to the lysogenic action of all the indicator strains were each different but strains 4 and 5 gave the same pattern. On all these strains lysis was due to heat sensitive phages. On strain 2 lysis was produced by the heat resistant phage as well as the heat sensitive phage of all the other indicator strains. The six groups were thus recognisable by these patterns. The heat sensitive phages responsible for the typical patterns of the indicator strains of groups 2 to 6 were isolated. From strains 9 and 12 (group 4) a moderately heat resistant phage was detected in the heated crude phage preparations P9/2 and P12/2. Other strains yielded phages much more sensitive to heat. The characteristics of these phages will be given in a separate paper.

DISCUSSION.

The 15 strains of S. *potsdam* were not readily grouped on lysogenicity or lysis patterns and appeared to represent many different bacteriophage groups characterised by carriage of different phages. Three major subdivisions were found consisting of two strains (2 and 6) in which no phage was detected, five strains (3, 10, 11, 15 and 17) in which heat resistant phage was not detected but heat sensitive phage of various lysis patterns was found, and eight strains (4, 5, 8, 9, 12, 13, 14 and 16) in which heat resistant phage was detected, usually accompanied by heat sensitive phage. The strains carrying only heat sensitive phage have not yet been grouped, but work is proceeding on the characterisation of their phages. Among the eight strains carrying heat resistant phage five groups (2-6) were distinguished on lysogenicity patterns on the

eight strains which were not lysed by the heat resistant phages which they carried. These lysogenicity patterns were therefore produced by the heat sensitive phages. Thus, when tested among themselves, these eight strains behaved as though they had no heat resistant phage. Inclusion of the nonlysogenic sensitive strains 2 and 6 in the test, however, showed up the heat resistant phage as well as any other phage. In the set of indicator strains proposed to detect the definable groups 2-6, with heat resistant phage, strain 2 was therefore included for the detection of strains, such as 4, carrying only heat resistant phage and therefore failing to lyse any of the other indicator strains. No more than two strains were placed in any one group. In group 1 were placed the nonlysogenic strains 2 and 6. The eight strains carrying heat resistant phage were divided into group 2 (strain 4), group 3 (strains 5 and 8), group 4 (strains 9 and 12), group 5 (strains 13 and 14) and group 6 (strain 16). Groups 2 to 6 were characterised, not only by their lysogenicity patterns on the indicator strains, but also by their failure to lyse with phage P5h/2.

Little connection appeared between the sources of the strains and their groupings. Strains 5 and 8, which came from different parts of the same animal, were both placed in group 3. Of the six lizard strains 4, 13, 14, 15, 16 and 17 from Queensland, strains 13 and 14 were similar and placed in group 5, strain 9 was placed in group 4 with a chicken strain (12), strain 16 was placed in group 6 and strains 15 and 17, which carried only heat sensitive phage, were ungrouped but appeared different from each other. The lizard strains were therefore heterogeneous. Of the four human strains 2, 3, 4 and 6, strains 2 and 6 were nonlysogenic and placed in group 1, strain 4 was placed in group 2, but strain 3, which carried only heat sensitive phage, was ungrouped.

Some of the characteristics of the heat resistant phages isolated from strains 4, 5, 8, 9, 12, 13, 14 and 16 have been investigated and are described in a separate paper (Atkinson and Bullas, 1956c).

Though only 15 strains of *S. potsdam* were investigated, they offered a wide variety of lysis and lysogenicity patterns dependent upon a wide variety of carried phages, both heat resistant and heat sensitive.

SUMMARY.

Fifteen strains of *S. potsdam* were examined for lysogenicity in pairs in all possible combinations. Two strains failed to produce any phage. The other 13 strains were all lysogenic. Five of these strains produced only heat sensitive phage and the other eight strains produced heat resistant phage frequently accompanied by heat sensitive phage. Eight heat resistant phages were isolated from these strains, all of which were resistant to lysis by the eight phages. Carriage of heat resistant phage was therefore correlated with failure to lyse with heat resistant phage.

The eight strains carrying heat resistant phage were placed in five groups (2-6) on the lysogenicity and lysis patterns shown among themselves. As they were all resistant to heat resistant phage, these patterns reflected the heat sensitive phages which they carried. The two strains in which phage was not found were allotted to group 1 and were sensitive to phage from all the other strains.

The six groups 1 to 6 were thus provisionally defined in terms of lysis and lysogenicity patterns determined on six indicator strains representing the six groups.

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FURTHER ASPECTS OF BEMEGRIDE ACTIVITY AND ITS RELATION TO BARBITURATE ACTION

by A. SHULMAN[1]

(From the Department of Physiology, University of Melbourne).

(*Accepted for publication 27th July, 1956.*)

A new barbiturate antagonist, Bemegride (I) (β-methyl-β-ethylglutarimide, Megimide, NP13) has recently been introduced into medical practice for the treatment of acute barbiturate intoxication (Shaw, Simon, Cass, Shulman, Anstee and Nelson, 1954; Shulman, Shaw, Cass and Whyte, 1955; Harris, 1955; Shulman, 1956) and is at present the focus of much active clinical and pharmacological research.

The specific nature of the antagonism of Bemegride to the barbiturates, (II) suggested by previous work (Shaw *et al.*, 1954; Shulman *et al.*, 1955), raised the question of its analeptic activity against such structurally related hypnotics as the monoureides and thiazolidine-2:4-diones.

This paper considers such analeptic activity and also presents evidence concerning the analeptic activity of the hydrolysis products of Bemegride, namely, β-methyl-β-ethylglutaric acid (VII) and β-methyl-β-ethylglutaramic acid (IV). Some further pharmacological properties of Bemegride are also indicated.

The pharmacological activity exhibited by Bemegride in animals and man generally depends upon the quantity and rate of its administration. Slow intravenous infusion usually produces asymptomatic awakening even from very deep barbiturate narcosis. This has been shown in dogs under EEG control (Shaw *et al.*, 1954) and has been confirmed in the mouse, rat, rabbit, cat, phalanger, sheep and man. Rapid intravenous administration to such deeply narcotised dogs produces, both clinically and electroencephalographically, a pattern of cerebral excitation followed by convulsions. After recovery from their convulsion, these dogs often relapse into a state of deep coma from which they may subsequently be awakened by further slow intravenous administration of Bemegride. Signs of cerebral excitation have likewise been produced in man by the administration of Bemegride (Shulman *et al.*, 1955).

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[1] This work has been supported by grants from the Australian National Health and Medical Research Council and from Nicholas Pty. Ltd. (Aust.).

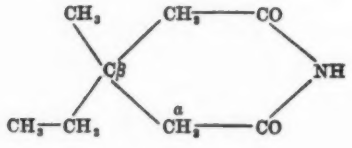
Bemegride does not invariably produce complete arousal. Thus, the cat usually cannot be fully awakened from barbiturate narcosis and attempts to do so frequently result in the production of toxic manifestations (Shaw *et al.*, 1954). The rabbit, on the other hand, can be readily awakened and put to sleep by the alternate intravenous administration, often of approximately equimolar concentrations, of Bemegride and thiopentone sodium in a manner suggestive of specific displacement.

Methods.

Coloured female mice of mixed strain weighing approximately 30 gm. were divided by random selection into test groups. The analeptic potency of Bemegride was estimated from its ability to reduce the sleeping time of mice given a prior injection of a selected hypnotic (pentobarbitone sodium, Carbromal, Bromural or 5-phenyl-thiazolidine-2:4-dione). The sleeping time was considered as the interval elapsing between the loss and complete recovery of the righting reflex. All drugs were administered intraperitoneally either in aqueous solution or, where necessary, in a 1 p.c. tragacanth suspension. The administration of the test substance was repeated at 10 minute intervals. The control group received saline in an analogous manner. A "t" test for significance was carried out in all cases.

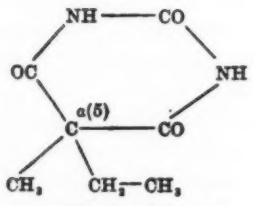
Throughout this paper, the carbon atom immediately adjacent to the carbonyl group in substances containing the -CO-NH-CO- (or CS-NH-CO) grouping is referred to as α. The carbon atom, next but one, to the carbonyl group is referred to as β. Thus, the α carbon atom in the barbiturate ring system corresponds to the 5 position of conventional nomenclature.

I



β-methyl-β-ethylglutarimide
(Bemegride, Megimide, NP13)

II



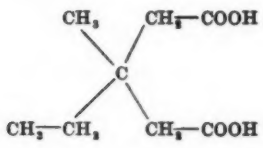
α-methyl-α-ethyl
(5:5)-barbituric acid

EXPERIMENTAL.

The Comparative Analeptic Activities of Bemegride, β-Methyl-β-Ethylglutaric Acid and β-Methyl-β-Ethylglutaramic Acid.

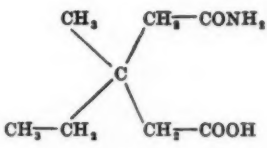
The comparative ability of these substances to reverse narcosis induced by pentobarbitone sodium (60 mg./kg.) was assessed. The results indicated in Table 1 show that neither β-methyl-β-ethylglutaric acid nor β-methyl-β-ethylglutaramic acid was an effective analeptic drug. This supports the work of Somers (1956).

III



β-methyl-β-ethylglutaric acid

IV



β-methyl-β-ethylglutaramic acid

TABLE 1.

	Control Saline	Bemegride (10 mg./kg.)	β-methyl-β-ethyl-glutaric acid (50 mg./kg.)	β-methyl-β-ethyl-glutaramic acid (50 mg./kg.)
No. of animals	31	28	29	26
Mean sleeping time (mins.)	67·6	55·7	86·6	86·3
Standard error (S.E.)	4·92	3·04	7·04	6·51
t-tests				
vs Saline		4·28***	0·68 N.S.	0·73 N.S.
vs Glutaric acid		3·98***		
vs Glutaramic acid		4·36***	0·03 N.S.	

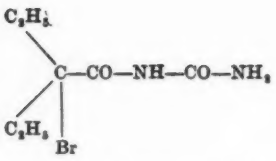
*** = highly significant ($p < 0·001$).
N.S. = not significant.

The Reversal of Monoureide Narcosis by Bemegride.

As it seemed that the intact ring structure of Bemegride was essential for effective barbiturate antagonism, experiments were conducted to assess the analeptic activity of Bemegride against the hypnotic action of the monoureides where the barbiturate ring system was no longer present. The drugs used were Carbromal (α-bromo-α-ethyl-butyrylurea (V)) and Bromural (α-bromo-β-methyl-butyrylurea (VI)). All drugs were administered intraperitoneally, Bemegride in aqueous solution and the two narcotics in a 1 p.c. tragacanth suspension. Table 2 indicates the value of Bemegride in antagonising the action of these monoureides in mice.

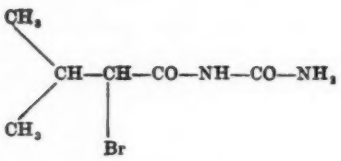
It has been further observed that Bemegride will antagonise the hypnotic and respiratory depressant properties of the monoureide α-phenyl-butyrylurea (phenyl-ethyl-acetylurea (VII)) (500 mg./kg.), administered intraperitoneally in a 1 p.c. tragacanth suspension to mice. However, the antagonism was less effective than that exhibited to either Carbromal or Bromural and the animals frequently regressed and often died.

V



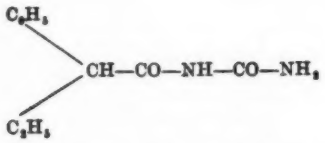
Carbromal (α-bromo-α-ethyl-butyrylurea)

VI



Bromural (α-bromo-β-methyl-butyrylurea)

VII

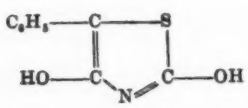


α-phenyl-butyrylurea (Phenyl-ethyl-acetylurea)

The Antagonism of Bemegride to the Hypnotic Action of 5-Phenyl-Thiazolidine-2:4-Dione.

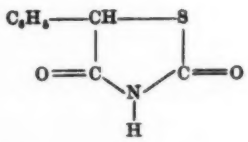
As prior work by Erlenmeyer (1938) and Marshall and Vallance (1954) had demonstrated the barbiturate-like narcosis produced by 5:5-disubstituted thiazolidine-2:4-diones, an investigation was conducted to assess the value of Bemegride in antagonising the hypnotic action of 2:4-dihydroxy-5-phenylthiazole (VIII) (Davies, MacLaren and Wilhemson, 1950) which in its tautomeric keto form may be written as 5-phenyl-thiazolidine-2:4-dione (IX). This substance forms a monosodium salt.

VIII



2 : 4-dihydroxy-5-phenyl-thiazole

IX



5-phenyl-thiazolidine-2 : 4-dione

Table 2 indicates that the moderate narcosis induced in mice by the intraperitoneal administration of an aqueous solution of the sodium salt of 5-phenyl-thiazolidine-2:4-dione (330 mg./kg.) was effectively antagonised by Bemegride.

The further pharmacological properties of 5-phenyl-thiazolidine-2:4-dione will be the subject of a separate paper (Shulman, 1956a).

TABLE 2.

	5-phenyl-thiazolidine-2 : 4-dione (sodium salt) (330 mg./kg.)		Carbromal (300 mg./kg.)		Bromural (350 mg./kg.)	
Injection: Saline	+	+	+	+	+	+
Bemegride	–	10 mg./kg.	–	20 mg./kg.	–	20 mg./kg.
Number of animals	18	18	24	23	24	25
Mean sleeping time (minutes)	76·4	23·2	57·5	29·4	67·0	34·6
Standard error (S.E.)	5·55		4·16		4·86	
"t" test vs Saline	9·66***		6·77***		6·67***	

*** = highly significant ($p < 0{\cdot}001$).

DISCUSSION.

This paper has provided additional evidence suggesting that Bemegride may antagonize barbiturate narcosis by a selective specific displacement mechanism. This is indicated by the approximately reversible stoichiometric titratability of Bemegride and thiopentone sodium often found in the rabbit and is further supported by the ability of Bemegride to reverse narcosis induced in mice by substances structurally related to the barbiturates, namely, the open chain monoureides and the thiazolidine-2:4-diones.

Strong supportive evidence is also obtained from a consideration of the case history of a patient suffering from chronic barbiturate intoxication who was treated with Bemegride (Palmer, 1956). This patient's tissues were heavily loaded with barbiturates as indicated by the prolonged and high dosage of these drugs ingested prior to Bemegride treatment. Yet, despite this, the administration of a comparatively small dose of Bemegride produced a quick and maintained reversal of the neurological signs and EEG findings characteristic of chronic barbiturate intoxication. Unfortunately, no blood, cerebrospinal fluid or urinary barbiturate concentrations were recorded in this case immediately prior to and following the administration of Bemegride.

The basis of this selective specific antagonism, from the pharmacological evidence available in this paper, may be found in the chemical relationships of the two groups of substances. The factor common to Bemegride and the barbiturates and related substances (monoureides, thiazolidine-2:4-diones) is

the presence of the -CO-NH-CO- grouping (or the CS-NH-CO grouping in the thiobarbiturates) associated with a mono- or disubstituted carbon atom in a position α or β to it. α substitution is seen in the barbiturates (II), the monoureides (V), (VI), (VII) and thiazolidine-2:4-diones (IX); β substitution is seen in Bemegride, (I). It has been shown that the analeptic activity of Bemegride disappears following the destruction of the integrity of the -CO-NH-CO- grouping (β-methyl-β-ethylglutaric and glutaramic acids, (III) and (IV), are inactive).

Consequently, it would appear that the basis of such a mechanism of selective specific displacement might reside in a competition of the analeptic and hypnotic drugs for receptor sites, one suited to the -CO-NH-CO- or modified CS-NH-CO grouping (this would be a pharmacologically passive attachment) and two others, producing pharmacologically opposite effects, so disposed as to respond to the presence of the substituent groups on the α and β carbon atoms respectively. The selectivity of the attachment of such drugs may depend principally on the steric configuration of the substituent groups attached to the α and β carbon atoms in fitting correctly into their receptor sites.

An extension of this hypothesis, indicating the importance of specific steric configuration as a basis for stimulant, analeptic, narcotic and anticonvulsant activity by these types of drugs, will be the subject of a separate paper now in preparation.

SUMMARY.

Bemegride, which antagonizes narcosis induced in mice by barbiturates (e.g., Pentobarbital sodium) also antagonizes the hypnotic action of the structurally related monoureides (e.g., Carbromal, Bromural) and thiazolidine-2:4-diones (e.g., 5-phenyl-thiazolidine-2:4-dione).

The hydrolysis products of Bemegride, β-methyl-β-ethylglutaric and glutaramic acids, have no analeptic activity.

Evidence is presented suggesting that this analeptic activity may be due to a selective specific site competition which may depend upon three receptor sites, one of which anchors the -CO-NH-CO- grouping which is common to all these drugs. The others, producing pharmacologically opposite effects, are so disposed as to respond to the substituent groups on the carbon atoms α or β to the -CO-NH-CO- grouping.

Acknowledgments. I am indebted to Nicholas Pty. Ltd. (Aust.) for their support in this investigation and for their generous gifts of Bemegride, 5-phenyl-thiazolidine-2:4-dione and β-methyl-β-ethylglutaric and glutaramic acids.

I wish to express sincere thanks to Miss Glenda M. Laycock and Mr. M. Anderson for technical assistance.

Grateful acknowledgment is made to Dr. K. H. Pausacker for many suggestions which contributed to the development of the hypothesis of drug action, to Mr. J. W. Legge for his helpful criticism, and to Professor R. D. Wright for his advice and guidance during this investigation.

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THE ACCURACY OF EOSINOPHIL COUNTS

by BRYAN HUDSON[1] and F. E. BINET[2]

(From the Baker Medical Research Institute and Alfred Hospital Clinical Research Unit, Melbourne, and the Department of Statistics, University of Melbourne).

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The object of this study was to investigate the errors associated with eosinophil counting. Similar investigations have been performed by Berkson and his colleagues (1936, 1939) in relation to the enumeration of red cells and leucocytes and by Rud (1947) who has analysed the errors associated with the counting of eosinophils. Berkson obtained a formula in which the variance in a series of haemocytometer counts was expressed in terms of variance components. From this formula he derived the estimate of variance as a linear function of the number of cells counted, haemocytometer chambers and pipettes used. The latter author derived a formula for the estimate of error which took the form of an exponential function.

Under the conditions of this series of experiments neither formula appeared to be applicable to the data. The purpose of this publication is to suggest that distributions other than that derived by Berkson may be more suitable for fitting to large series of estimates of red or white cell concentrations, when errors such as observer fatigue may appear.

Materials and Methods.

Samples of blood were taken from the antecubital veins of healthy volunteers and hospital patients. Coagulation was prevented by the addition of heparin. The eosinophil counts ranged from 110 to 5,100 cells per cubic millimeter of blood.

After the sample had been manually shaken for two minutes to ensure thorough mixing, ten white cell pipettes were loaded, first with blood and then diluting fluid to give a final dilution of 1 : 20. The pipettes were shaken for about thirty seconds and allowed to stand for at least twenty minutes, after which they were shaken mechanically for two minutes. The diluted blood was then run into one chamber of a Fuchs Rosenthal haemocytometer (0·2 mm. deep). After the loaded haemocytometer had stood for at least ten minutes all the cells in the ruled area (16 sq. mm.) were counted, using a microscope with a low power objective and × 20 oculars.

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[1] This investigation has been carried out in partial fulfilment of the requirements of the University of Melbourne for the Degree of Doctor of Philosophy.

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The diluting fluid used was a mixture of 1 p.c. phloxine in 50 p.c. aqueous propylene glycol and 1 p.c. sodium carbonate. In this alkaline medium all leucocytes other than eosinophils underwent lysis (Pilot, 1950).

All counts were performed by the same person in the one working day.

RESULTS.

These are shown in Table 1. Each figure represents the number of cells counted from one pipette delivered into one chamber (3·2 cu. mm.).

TABLE 1.

Number of cells counted (3·2 *cu. mm*).

Pipette No.										$\bar{r}$	s^2	1/t
1	2	3	4	5	6	7	8	9	10			
23	16	23	12	15	20	16	25	15	14	17·9	20·10	·006866
25	23	18	31	29	22	30	18	19	20	23·5	25·17	·003018
28	34	45	34	25	35	22	34	38	29	32·4	44·27	·011304
43	44	29	29	34	33	24	43	37	39	35·5	47·17	·009257
45	20	32	34	39	41	37	43	31	34	35·6	52·04	·012975
47	42	36	36	37	30	41	26	36	27	35·8	44·40	·006710
23	39	38	36	41	48	40	35	39	45	38·4	44·49	·004129
38	42	36	43	42	34	53	53	53	42	43·6	50·49	·003624
57	60	63	57	58	56	74	83	60	72	64·0	84·00	·004883
47	58	72	68	83	70	90	67	79	52	68·6	182·71	·024248
96	79	85	95	98	73	90	60	62	103	84·1	229·43	·020548
114	88	95	108	103	106	95	111	107	122	104·9	101·43	—·000315
99	89	116	109	85	97	120	112	131	106	106·4	202·71	·008507
115	123	88	138	101	118	97	104	130	119	113·3	242·68	·010079
105	122	132	121	115	114	137	119	137	113	121·5	115·61	—·000399
138	124	119	118	132	115	124	98	129	114	121·1	124·32	·000220
142	136	123	155	160	144	134	140	157	143	143·4	129·82	—·000660
123	176	140	154	165	158	157	178	152	152	155·5	260·94	·004361
155	207	206	205	179	176	211	201	224	202	196·6	415·38	·005660
234	247	268	163	236	227	225	239	233	256	232·8	777·29	·010047
231	226	237	230	265	271	266	242	271	254	249·3	367·12	·001896
349	403	391	338	330	324	405	415	354	376	368·5	1136·72	·005657
771	746	811	951	848	860	793	822	790	905	829·7	3935·57	·004512

DISCUSSION.

Inspection of Table 1 shows that sample variances increase with, and in most cases exceed, sample means. This observation contrasts with that of Berkson and his colleagues (1939) who found that sample variances were less than sample means. Their investigation, although similar to this one, was rendered somewhat artificial by their method of enumeration, in which they photographed the haemocytometer chambers and counted the cells present by an electronic device — circumstances which would rarely prevail in either routine or research laboratories.

The statistical treatment of data derived from such an investigation as this is based, traditionally, upon the assumption that the individual counts follow the Poisson frequency distribution. In the present investigation most of the sample variances exceed the sample means, a situation which casts doubt on such an assumption. Using a test of goodness of fit based on Cochran's (1936) coefficient of dispersion, a χ^2 value of 368 was obtained with 207 degrees of freedom. This is highly significant, indicating that the Poisson hypothesis is not appropriate for these data. There exists a variety of frequency distributions, related to the Poisson distribution, one or more of which may provide a better fit to the data. On *a priori* grounds there is no particular hypothesis which could be adduced to account for the above results; nor is it usually practicable to choose between these frequency distributions on statistical grounds unless there is an exceedingly large number of observations.

The fact that sample variances increase with sample means at a rate more rapidly than linear, is attributed to observer fatigue, but this assumption cannot be given a precise mathematical form. On the grounds of convenience, the binomial distribution was chosen as an alternative to the Poisson. This distribution, besides being one of the most simple of those related to the Poisson, has the additional advantage that it can be fitted to samples with variances either smaller or larger than the sample means. It may be written conveniently as

$$Pr\,(r) = \binom{\theta+r-1}{r}\left(\frac{\mu}{\mu+\theta}\right)^r\left(\frac{\theta}{\mu+\theta}\right)^\theta,$$

where $Pr\,(r)$ is the probability of obtaining a count of value r, the population mean count being μ and the variance

$$\mu + \frac{\mu^2}{\theta}.$$

As a further simplification it is assumed that θ is constant throughout, while μ varies from sample to sample. From a single sample μ and $1/\theta$ may be estimated by computing:

$$m = \bar{r},$$

$$1/t = \frac{s^2 - \bar{r}}{\bar{r}^2}$$

where m, t are the estimates of μ and θ, respectively, $\bar{r}$ is the arithmetic mean of all counts in a sample and $s^2 = \Sigma(r-\bar{r})^2/N-1$, with summation over all counts in the sample, and N the number of counts summed. (These are given in the last two columns of Table 1.)

These estimates are not the most exact obtainable (Bliss and Fisher, 1953), but any attempt to increase the accuracy would not be justified in view of the complexity of the calculations on data of this nature (Anscombe, 1950).

Since μ is assumed to vary from sample to sample, each m is an estimate of a distinct parameter. By contrast, it is assumed that θ has a common value, which implies that the twenty-three values of $1/t$ differ from each other by chance only. This latter assumption was subjected to a χ^2 test, which did not indicate the existence of any inhomogeneity ($\chi^2 = 22{\cdot}7$, with 22d.f). Consequently the weighted mean of the several estimates was calculated, yielding 296·8 for the estimate of θ. A test of goodness of fit, analogous to that used earlier, was applied to this distribution yielding a χ^2 of 248 with 206 degrees of freedom which is significant at the 5 p.c. level. However, the original χ^2 has now been reduced by 120 for a loss of 1 degree of freedom; this highly significant reduction is an indication of the superior fit of the negative binomial distribution. It is unlikely that the conclusions drawn below would have been materially altered by refining the hypothesis further in order to obtain a closer fit. Consequently, we assume that the significant χ^2 is due to random variation, in excess of the variability accounted for by the binomial model, and will increase our estimates of variance accordingly.

The problem of the accuracy of eosinophil counts may now be considered on the basis of the foregoing assumptions and estimates. The estimated probability of obtaining a count r when the mean is μ, is

$$\left(\frac{295{\cdot}8+r}{r}\right)\left(\frac{\mu}{\mu+296{\cdot}8}\right)^{r}\left(\frac{296{\cdot}8}{\mu+296{\cdot}8}\right)^{296{\cdot}8},$$

and the estimated variance of such a count would be

$$\mu\,(1+0{\cdot}003369\,\mu)$$

on the proper binomial model. To take the significantly high χ^2 into consideration, we multiply this estimate by an inhomogeneity factor (Finney, 1952),

$$f = \chi^2/\nu = 248/206 = 1{\cdot}204,$$

and use this inflated value for further computations.

On approximating to this distribution by a normal one with the same mean and variance, Table 2 is obtained, showing the mean μ and the percentage p such that the odds are approximately 19 to 1 that a single count will not deviate from μ by more than $p\%$ of μ. For example, if the true count is 100, the odds are approximately 19 to 1 that a single count will fall between 75 and 125.

TABLE 2.

μ	10	25	50	100	250	500	1,000
p	69·1	44·8	32·9	24·9	18·4	15·8	14·3

The fact that the counts in the above experiment behave like samples from a family of distributions where the variance exceeds the mean, while the counts in Berkson's (1939) experiment appear to come from a family of distributions

where the variance equals the mean, may originate from the elaborate method used by Berkson to eliminate observer fatigue. The expression for the coefficient of variation, obtained by Berkson, can be rearranged to give the estimated variance of a count. Using the values appropriate to the experimental conditions, the resulting variance estimate would be

$$\mu(1 + 0{\cdot}003267\mu),$$

which is of the same form as the estimate given above. The smaller coefficients arise from Berkson's photographic method but where counts have to be performed without such techniques, it seems realistic to assume that observer fatigue will appear. Under such circumstances, it might be advisable to follow a procedure of repeated counts similar to that reported here so as to ascertain whether a family of over-dispersed distributions would fit the observations.

The actual form of the distribution and the numerical values of its parameters may well differ from one laboratory to another, and from one type of particle count to another.

SUMMARY.

The accuracy of eosinophil counts has been investigated.

It is suggested that the assumption that such counts, performed under standard laboratory conditions, follow the Poisson frequency distribution is not a valid one since the sample variances consistently exceed the sample means.

The negative binomial distribution has been fitted to the data and found to yield a convenient alternative frequency distribution.

In Table 2, the accuracy of an estimate of an eosinophil count is given.

It is suggested that this type of distribution may apply to the enumeration of other cells in the blood.

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A STUDY OF THE INHIBITORY ACTION OF ADRENALINE

1. EFFECTS ON THE CARBOHYDRATE METABOLISM OF ISOLATED RABBIT INTESTINE[1]

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Since the classical studies of Barger and Dale in 1910, there have been many papers published on the pharmacology of the sympathomimetic amines. Much work has also been done on the effects of adrenaline on the carbohydrate metabolism of such tissues as skeletal muscle, liver, diaphragm, etc. In 1934, Hegnauer and Cori showed that very low concentrations of adrenaline caused the breakdown of glycogen, and the accumulation of hexosemonophosphate in frog skeletal muscle. Later, Cori and Cori (1936a, 1936b) showed further effects of adrenaline on phosphorylation mechanisms. Since then various aspects of this problem have been investigated by Wajzer (1938), Cohen (1949), and Ellis and his co-workers (Ellis and Anderson, 1951; Ellis, 1955) amongst others. However, to explain the effects of adrenaline on smooth and cardiac muscle at a biochemical level is a more difficult problem. Ellis (1952) showed that the excitatory effects of adrenaline on frog heart are maintained under anaerobic conditions, or in the presence of metabolic inhibitors such as iodoacetate, only if an adequate supply of glucose is available. Lichtneckert and Straub (1949) suggested that adrenaline catalysed the formation of adenosine triphosphate or its precursor in heart muscle. The most exhaustive study of the biochemistry of the inhibitory effect of adrenaline comes from Mohme-Lundholm (1953). This worker claimed that when tissues such as isolated rabbit intestine were bathed in Tyrode solution containing a large excess of sodium bicarbonate, their responses to adrenaline were considerably reduced. A similar reduction, or even abolition of the response, was found when the tissues were treated with metabolic inhibitors such as copper or fluoride. Also, Mohme-Lundholm reported an increase in the lactic acid concentration of intestine which had been relaxed with adrenaline. On the basis of these findings, she proposed the fol-

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lowing theory to explain the inhibitory effects of adrenaline: the addition of the hormone causes an increased production of lactic acid, and the consequent lowering of the intracellular pH causes the smooth muscle to relax.

However, these conclusions may be criticised. The addition of a large amount of $NaHCO_3$ to the perfusing fluid will alter not only the buffering capacity of the fluid, but will also increase the osmotic pressure and raise the pH. Hence it may be unwise to expect identical responses from tissues under such diverse conditions. The addition of metabolic inhibitors might well affect not only the metabolism of the muscle, but the contractile mechanism itself and in fact, both copper and fluoride ions readily produce an irreversible spasm of isolated intestine. And finally, an increase in the lactic acid content of the relaxed intestine could possibly be incidental to the processes which actually caused relaxation.

Therefore, experiments were commenced to investigate the following points: (i) is the response of the isolated rabbit intestine to adrenaline altered by bathing it in a modified Tyrode solution in which all the sodium chloride is replaced by equimolar sodium bicarbonate; (ii) can an increase in the lactate concentration of the isolated intestine be detected when the intestine is caused to relax fully with adrenaline; (iii) can any effect on the response of the isolated intestine be detected when it is bathed in modified Tyrode solutions, containing in place of glucose, lactate or other possible sources of energy.

Methods.

Young male rabbits were used throughout. They were stunned and bled to death. A portion of the small intestine about 45 cm. long was removed, starting a few centimetres below the stomach. This was washed carefully with Tyrode solution, and placed on filter paper wetted with Tyrode solution, in a petrie dish. Pieces about 2 cm. long were taken from the central region and suspended in an organ bath of 10 ml. capacity at 37° C. The piece of intestine was attached with cotton to a side-writing, gimbal-mounted lever, and the trace was recorded on a conventional kymograph. The preparation was aerated with oxygen containing 5 p.c. carbon dioxide. It was allowed to equilibrate for at least 30 minutes, and it was then treated with repeated doses of adrenaline until its response was constant. Under these conditions preparations were obtained which gave a regular, even beat. They would respond with a reduction in the height of the beat to doses of adrenaline down to 0·05 μg., and doses of 0·5-1·0 μg. usually caused complete loss of tonus and cessation of beat for at least 1 minute.

In the experiments testing the effect of raised bicarbonate the procedure was as follows. After equilibration, an approximate dose-response curve was constructed, using amounts of adrenaline which would cause approximately 25, 50 and 100 p.c. inhibition of the beat. The intestine was then bathed with a modified Tyrode solution, containing, in place of the usual 8 gm./l. of sodium chloride, 11·48 gm./l. of sodium bicarbonate. The pH of this solution after bubbling for 20 minutes with "carbogen", was 8·0. After 20 minutes equilibration in this solution, which was changed frequently, the responses of the preparations to adrenaline were determined again. When these responses had become con-

stant, the preparation was returned to normal Tyrode, and after a further 20 minutes equilibration, the responses were determined again.

Lactic acid was determined by the method of Barker and Summerson (1941). To determine the accuracy of this method, a calibration curve was prepared and its regression equation calculated. This was $X = 76 \cdot 71\ Y + 0 \cdot 39$. The error mean square (12 degrees of freedom) was 0·8, i.e. in the central region of the curve, lactic acid concentration can be estimated with an error not exceeding 5 p.c.

Experiments on lactic acid production by intestine were divided into two sections:

(a) Preliminary experiments were run in the attempt to demonstrate increased lactic acid in the fluid bathing the intestine, subsequent to the addition of adrenaline. Pieces of intestine were set up as described above. After the customary period of equilibration, the preparation was given a final wash out. Exactly 2 minutes after this wash, 4·5 ml. of Tyrode solution from the organ bath was taken by pipette, and the lactic acid content determined. This sample was the control. The preparation was again washed out several times, and 1·5 minutes after the last wash, a dose of adrenaline was added, giving full relaxation. 30 seconds later, an aliquot of 4·5 ml. was again taken from the bath. This sample was the test. Further alternate controls and tests were taken, as well as samples of fluid taken from the bath while the intestine was relaxed by the addition of sulphuric or phosphoric acids.

(b) The main experiments in this section were devoted to assaying the intestine itself. The procedure was as follows: After the intestine had been removed from the rabbit as described above, 4 to 6 adjacent pieces about 2 cm. long were cut off from the region of the ileum. These were carefully blotted with filter paper, both inside and out, and then placed in weighed flasks of Tyrode solution. These were weighed again, and then held at room temperature until required. Each piece of intestine was in turn set up in the organ bath at 37° C., bubbled with "carbogen" and equilibrated with frequent washouts for 30 minutes. Exactly two minutes after the last wash, the tissue was lifted from the bath, and dropped into a mortar containing 2 gm. of washed sand and 1 ml. of 10 p.c. trichloracetic or phosphotungstic acid. It was ground as quickly as possible, and further 10 p.c. trichloracetic or phosphotungstic acid added to 10 ml. By this technique, the tissue was completely disrupted in 10 to 15 seconds after its removal from the bath. The resultant fine suspension was allowed to stand at room temperature for 30 minutes, with frequent stirring, and was then centrifuged. Five millilitre aliquots of the clear supernatant were taken for assay. Pieces treated in this way were the control experiments. Alternate pieces of tissue from the same intestine were treated with adrenaline in the following manner. After equilibration in the organ bath at 37° C. for 30 minutes, as for the controls, these pieces were given two "priming" doses of adrenaline, sufficient to cause complete loss of both tone and rhythmic beating for at least 2 minutes. The amount used was usually 1·0 μg., but was sometimes as low as 0·5 μg., giving a final concentration of 1 in 2×10^7. Exactly 90 seconds after the second "priming" dose of adrenaline had been washed out, a "test" dose (0·5 to 1·0 μg.) of adrenaline was added. Exactly 30 seconds later, while the muscle was completely relaxed, it was taken from the bath, and extracted with phosphotungstic or trichloracetic acid, as described above. The technique of "priming" the tissue with adrenaline was used, as it has been found that the intestine's response to the first dose of adrenaline is always smaller than the subsequent responses, and after contact with adrenaline, the beat is usually much improved.

Minor variations in this procedure were introduced. In some experiments, the tissue was allowed to remain in contact with adrenaline for 3 minutes. By this time, the muscle was just beginning to recover its tone. In another experiment, the concentration of adrenaline was raised to $1 : 10^6$, and in another, the effect of calcium chloride (0·4 ml. of 1 p.c.) was tested. In addition, identical experiments were run on pieces of intestine perfused with

2 : 4-dinitrophenol, and with phlorrhidzin. Dinitrophenol was chosen as it is known to increase the lactic acid production of many tissues (see, for instance, McIlwain and Gore, 1951). It has been found that a concentration of up to $1 : 2 \times 10^5$ ($1 \cdot 3 \times 10^{-4}$M) could be tolerated by the intestine without any serious reduction in the amplitude of its beat. Phlorrhidzin was chosen as a metabolic inhibitor acting at an early stage of the glycolytic cycle (see Shapiro, 1947). The intestine can tolerate concentrations of 1 in 10^4 without visible effect. Neither drug had any effect on the intestine's response to adrenaline.

The recovery of lactic acid from intestine was investigated as follows: From each of two intestines, four pieces of tissue were cut off, as described above. After careful washing with Tyrode solution, the four control pieces were assayed for lactic acid. Alternate pieces of intestine were used as the test experiment. Immediately after they had been ground with phosphotungstic acid, 200 μg. of lactic acid were added, and they were then assayed in a manner identical with the controls.

In experiments designed to test the effect of various substrates on the intestine's response to adrenaline, the procedure used was as follows. A piece of intestine was set up in the organ bath in the usual manner, in normal Tyrode solution. After the tissue had been equilibrated, a dose-response curve was determined, using amounts of adrenaline giving 25, 50 and 100 p.c. inhibition of the beat. The intestine was then perfused with Tyrode solution containing no glucose or other organic material (this solution will hereafter be referred to as "inorganic Tyrode"). After varying times in the "inorganic Tyrode" the beat failed progressively, until it was but a small fraction of the original amplitude in glucose. Having so little tone, even large doses of adrenaline were unable to cause any further relaxation. The exhausted intestine was then perfused with one of a number of modified Tyrode solutions, containing some other organic material in place of glucose. If a recovery of the beat was observed, the intestine's responses to the same amounts of adrenaline were again determined. The tissue was then exhausted a second time in "inorganic Tyrode", and finally returned to glucose Tyrode. After the beat had recovered, the responses to adrenaline were tested once again. Where the substitute for glucose was in the form of a sodium salt (for example, sodium acetate, sodium lactate, etc.), a quantity of 1 gm./l. was arbitrarily chosen, and an equivalent reduction in the sodium chloride was made to maintain the original sodium ion concentration. The substances tested were as follows:

Sodium acetate	1 gm./l.
Sodium pyruvate	1 gm./l.
Sodium lactate	1 gm./l.
Sodium citrate	1 gm./l.
Sodium succinate	1 gm./l.
Sodium propionate	1 gm./l.
Sodium β-glycerophosphate	1 gm./l.
Glycerol	1 gm./l.
Glycine	1 gm./l.

RESULTS.

The effect of increased buffer capacity of the perfusing fluid.

It was found that the intestine's response to adrenaline was identical in normal Tyrode, and in Tyrode containing sodium bicarbonate in place of sodium chloride. This perfusing fluid contained in 10 ml., bicarbonate equivalent to 13·8 mg. lactic acid. Experiments described below have shown that the total

amount of lactic acid contained in such a piece of intestine is unlikely to exceed 0·5 mg. Hence, while the buffering capacity of the fluid used in this experiment is less than that of Mohme-Lundholm's work, it appears to be more than adequate to neutralise the lactic acid present, assuming that it is available within the cells.

The lactic acid content of intestine, and the effect of adrenaline on this.

(a) *Recovery of added lactic acid.*

In the four experiments, recoveries were found to be 86, 63, 86 and 80 p.c. (average, 79 p.c.).

(b) *The concentration of lactic acid in the fluid bathing the intestine.*

The results of two experiments, on two different intestines, are summarised in Table 1. It will be seen that, while the Tyrode solution contains small amounts of lactate, the addition of adrenaline, or of other substances which also relax the intestine, does not significantly affect the concentration of this metabolite.

TABLE 1.

Lactate, μg. in 10 ml. of fluid from the organ bath.

Rabbit No. 1		Rabbit No. 2	
1st control	9·0 μg.	1st control	13·7 μg.
In adr. $1 : 2 \times 10^7$	8·0 μg.	In adr. $1 : 10^6$	13·6 μg.
2nd control	13·3 μg.	2nd control	13·3 μg.
In adr. $1 : 2 \times 10^7$	13·3 μg.	In adr. $1 : 10^6$	10·7 μg.
3rd control	15·3 μg.	3rd control	13·6 μg.
In H_2SO_4 1 : 1500	18·0 μg.	In H_3PO_4, 1 : 1300	10·6 μg.
4th control	15·3 μg.	4th control	12·3 μg.
		In H_3PO_4, 1 : 750	13·3 μg.

As these results showed no effect of adrenaline, the tissue itself was assayed for lactate.

(c) *The concentration of lactic acid in rabbit intestine and the action of adrenaline on this.*

The results of 7 experiments, all on different intestines, are presented in Table 2.

It will be seen that in the control assays, variations in the lactic acid content of as much as 30 p.c. may be encountered between adjacent pieces of the same intestine, although usually agreement is closer than this. Variations between different intestines are much greater, average values ranging between 0·49 and 0·74 mg./gm. Statistical analysis shows that variations between the control and the adrenaline-treated tissues are not significant, and in fact, there appears to be no indication whatsoever that the lactic acid content is significantly raised during treatment with adrenaline.

TABLE 2.

Lactic acid (mg/gm. tissue).

	Control pieces of intestine	Intestine fully relaxed with adrenaline
Rabbit No. 1	0·50, 0·50, 0·65 } 0·55	0·45, 0·53 } 0·49
Rabbit No. 2	0·44, 0·55 } 0·49	0·45, 0·42, 0·35 } 0·41
Rabbit No. 3	0·89, 0·96, 0·98 } 0·94	0·93, 0·92, 1·26 } 1·04
Rabbit No. 4	0·72, 0·67, 0·73 } 0·71	0·70, 0·72 } 0·71
Rabbit No. 5	0·73, 0·64, 0·52 } 0·63	0·80, 0·66 } 0·73
Rabbit No. 6	0·73, 0·64 } 0·68	0·65, 0·53 } 0·59 (after 3′ in adrenaline recovery just beginning)
		0·60 (in calcium)
Rabbit No. 7	0·84, 0·65 } 0·74	0·91 (after 3′ in adrenaline recovery just beginning)
		0·47
		0·58 (in adrenaline 1 : 10^6)

TABLE 2A.

Statistical analysis of results of Table 2.

Sources of Variation	d. of f.	S. of Sq.	M. Sq.
Treatment eliminating rabbits	1	0·0011	0·0011 N.S.
Rabbits	6	0·9900	
Error	27	0·3341	0·012
Total	34	1·3252	

Table 3 presents results of assays on intestines perfused with phlorrhidzin 1 : 10^4 and with 2 : 4-dinitrophenol 1 : 2×10^5. Once again, it can be seen that there is variation within the control values, and from one intestine to another, but no significant effect of adrenaline. It is also interesting to note that at this concentration, dinitrophenol does not appear to raise the level of lactic acid over that of normal intestine.

TABLE 3.

	Lactic Acid (mg./gm. tissue)	
	Control of pieces of intestine	Intestine fully relaxed with adrenaline
A. *Phlorrhidzin*		
Rabbit No. 1	0·97, 0·78 } 0·87	1·03, 1·78 } 0·91
Rabbit No. 2	1·16, 0·96 } 1·06	1·04, 1·01 } 1·03
B. 2 : 4-*dinitrophenol*		
Rabbit No. 1	0·65, 0·83 } 0·74	0·86, 0·69 } 0·77
Rabbit No. 2	1·35, 0·83 } 1·17	1·10, 0·97 } 1·03

The ability of various substrates to support that beat of the isolated intestine, and the responses of such perfused intestines to adrenaline.

It was found that when the isolated intestine was bathed in Tyrode solution lacking all organic material, its beat began to fail after a variable time. Usually, after 30 to 45 minutes without glucose, the beat had fallen to a small fraction of its original amplitude. However, it was found that it was not possible completely to abolish the beat even after very prolonged perfusion without glucose. In this condition, since the intrinsic tone of the tissue was so low, only the smallest responses were noted to even large doses of adrenaline. After about 1 hour in the "inorganic Tyrode", the tissue was bathed with the modified Tyrode containing the substrate being tested. It was found that, of all the substrates tested, only lactate, pyruvate, and acetate were able to restore the beat. Full recovery of the beat usually took 15-20 minutes and occasionally a little longer with acetate. None of the other substrates tested were able to restore the beat, although it always returned very shortly after glucose-Tyrode was admitted to the bath.

It was found that pieces of intestine supported solely by lactate, pyruvate or acetate gave responses to adrenaline identical with those obtained in "glucose Tyrode".

After these responses had been established, the intestine was then returned to "inorganic Tyrode" and the beat allowed to fail again. To complete the experiments, glucose Tyrode was then admitted to the organ bath. After full recovery (which always occurred within a few minutes) the responses to adrenaline were checked again. It was found that in all cases, they did not vary significantly from the original responses.

It seemed probable that the tissue was resynthesising glycogen from lactate, acetate and pyruvate. This possibility was examined, using the technique of Seifter *et al.* (1950) in the following way: Three adjacent pieces of the same intestine were treated as follows:

(i) The first was suspended in oxygenated glucose-Tyrode at 37° C., and after the usual period of equilibration, it was removed and assayed for glycogen.

(ii) The second piece was equilibrated in glucose-Tyrode as above, and then allowed to exhaust in "inorganic Tyrode". It was removed after 30-45 minutes in this medium and assayed for glycogen.

(iii) The third piece was allowed to exhaust in "inorganic Tyrode" and then perfused with acetate-, lactate-, or pyruvate-Tyrode. When the beat had recovered, the tissue was removed and assayed for glycogen. It was found that the glycogen content of the tissue perfused with lactate, acetate and pyruvate was of the same order as that of intestine perfused with glucose, while the exhausted gut had a much lower value. It was not possible completely to deplete the tissue of glycogen, even after prolonged perfusion with "inorganic Tyrode". Hence it has been impossible to examine the response to adrenaline of an intestine producing the energy for its beat directly from the oxidation of lactate, pyruvate or acetate.

Attempts were made to modify the response of the intestine to adrenaline by treating it with high concentrations of insulin, which, in the intact animal, is known to have effects on carbohydrate metabolism directly opposite to those of adrenaline. Pieces of intestine were set up as usual in "inorganic Tyrode" and their responses to adrenaline were determined. They were then perfused with "inorganic Tyrode" containing insulin, 2 units/litre, and their responses to adrenaline were determined again. It was found that, even at this very high concentration insulin did not affect the response to adrenaline. Studies were also made on the effects of various drugs, known to inhibit steps in the catabolism of glucose. These drugs were: phlorrhidzin $1 : 10^4$, copper sulphate $5 : 10^5$, sodium fluoride $2 : 10^4$, sodium malonate $1 : 10^5$, sodium iodoacetate $1 : 10^5$ and dinitrophenol $1 : 10^5$. Copper and fluoride caused a very marked increase in the tone of the intestine, and an irregular beat. These effects occurred even when the compounds were added to the bath in divided doses of 100 μg. Phlorrhidzin at $1 : 10^4$ did not affect the tone of the gut, nor the amplitude of its beat. All the other drugs tested reduced the amplitude of the beat as much as 50 p.c. and therefore higher concentrations were not tested.

Copper produced some reduction in the response of the gut to adrenaline. However, it also caused a reduction in the response to lactic acid. This is interpreted as being due to an action of copper on the contractile mechanism of the intestinal musculature.

All the other drugs tested had no effect at all on the gut's response to adrenaline.

DISCUSSION.

The results presented in this paper are almost without exception at variance with those of Mohme-Lundholm. The gut preparations used in the present study were more sensitive to adrenaline than those of the Scandinavian worker, who appeared to require concentrations of 1 : 750,000 to obtain full inhibition of the intestine. In the present study, full relaxation was obtained with concentrations of $1 : 5 \times 10^6$ or less, and it seems possible that the increase in lactic acid concentration reported by Mohme-Lundholm was merely incidental to some other process which actually was the primary cause of relaxation. The method of extracting the lactic acid from the tissue used in the present work would produce a more rapid immobilisation of the enzymes than the method used by Mohme-Lundholm. Furthermore, Barker and Summerson's method of assaying for lactic acid, which was used in the present study is more sensitive than, and at least as reliable as, the method of Friedman and Graesser, which Mohme-Lundholm used.

The experiments using metabolic inhibitors are not conclusive. It is possible that the concentrations used were not adequate to block the postulated increase in lactate production. However, since several of these inhibitors (iodoacetate, dinitrophenol) produced about 50 p.c. inhibition of the beat of the intestine, it is reasonable to assume that they were interfering with some aspect of the energy production of the intestine. Hence, if adrenaline acts solely *via* an increased lactate production it is strange that these compounds did not affect the intestine's response to adrenaline. The only metabolic inhibitor which did reduce the gut's response to adrenaline was copper and since it sent the tissue into spasm, and at the same time reduced its response to lactic acid itself, copper is probably acting at some site other than the carbohydrate cycle, perhaps directly on the contractile mechanism.

It is unfortunate that it has not been possible to obtain a gut preparation which would beat under conditions where all the energy supply came from the oxidation of 2 or 3 carbon fragments of glucose breakdown. Isolated intestine, deprived of external glucose, rapidly exhausts its endogenous fuel, and its beat fails almost completely. When supplied with lactate, pyruvate, or acetate, it is able to resynthesise glycogen, and commence beating again. Hence, even though the tissue is supplied solely with lactate, etc., it appears to require glycogen to supply the energy for its beat.

The experiments using insulin again do not provide conclusive evidence. However, adrenaline and insulin have been known for many years to have antagonistic actions on carbohydrate metabolism in the intact animal, and the complete inability of very high concentrations of insulin to reduce the intestine's response to adrenaline, together with the other evidence presented, does appear to cast doubt on Mohme-Lundholm's theory.

An even more serious criticism of her hypothesis may be drawn from figures presented in her own paper. Here she claimed to show that doses of adrenaline which produced full inhibition of the intestine also produced an average increase in its lactic acid concentration of 36·8 p.c. However, doses of noradrenaline, also causing complete inhibition, produced an average increase in the lactic acid content of only 12·6 p.c. This finding is difficult to reconcile with a theory which postulates that the sole cause of the relaxing action of the sympathomimetic amines is the lactic acid whose production they stimulate.

SUMMARY.

Mohme-Lundholm's theory, claiming to explain the inhibiting action of adrenaline on isolated rabbit intestine, has been examined. The findings which she presents to support this theory are not confirmed.

No increase in the lactate content of isolated rabbit intestine has been found when the intestine was fully relaxed by adrenaline.

No alteration in the intestine's response to adrenaline could be produced by the addition of various inhibitors of carbohydrate metabolism, by high concentrations of insulin, or by an increase in the buffer capacity of the perfusing fluid.

Similar negative effects have been obtained from intestine preparations whose only source of energy was added lactate, pyruvate, or acetate. However, under these conditions, the tissue is able to maintain its normal glycogen levels.

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